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Description

This invention relates to the use of recombinant DNA technology for the production of hepatitis B surface antigen. In one aspect, the present invention relates to the production of hepatitis B surface antigen in Pichia. In another aspect, the present invention relates to novel DNA constructs encoding hepatitis B surface antigen. In yet another aspect, the present invention relates to novel organisms transformed with the above described DNA constructs.

Background

As recombinant DNA technology has developed in recent years, the controlled production by microorganisms of an enormous variety of useful polypeptides has become possible. Many eukaryotic polypeptides, such as for example, human growth hormone, leukocyte interferons, human insulin and human proinsulin have already been produced by microorganisms. The continued application of techniques already in hand is expected in the future to permit production by microorganisms of a variety of other useful polypeptide products. One such useful polypeptide product is hepatitis B surface antigen.

Hepatitis B (serum hepatitis) virus is transmitted among humans and manifests itself as chronically debilitating infections which can result progressively in severe liver damage, primary carcinoma, and, ultimately, death. In most cases, complete recovery from hepatitis B infections can be expected. However, large segments of the population, especially in many African and Asian countries, are chronic carriers with the dangerous potential of transmitting the disease pandemically.

Effective prophylaxis of the hepatitis B virus is to administer a hepatitis B virus vaccine which is usually a highly purified hepatitis B surface antigen. Such a hepatitis B virus vaccine is effective for preventing infection with the virus. Especially high risk groups are those people who need blood transfusions or dialysis treatment, medical personnel working with such groups, and the like. In addition, such vaccine is also effective for preventing generation of new carrier, and it may therefore be possible to eliminate completely the hepatitis B virus from earth.

The hepatitis B virus has not been infectious in cell culture and can, therefore, only be obtained from infected humans or higher primates. Thus, means have not been available for obtaining and maintaining sufficient supplies of hepatitis B virus for use in producing antigen for immunization against hepatitis B virus.

The hepatitis B virus vaccine is usually prepared by isolating and purifying hepatitis B surface antigen from blood plasma of hepatitis B virus carriers. Such purification, however, must be done extremely efficiently since only very low concentrations of the desired antigen are present in the plasma being purified. Hence, it has heretofore been very difficult to prepare the desired hepatitis B virus vaccine on an industrial scale.

Objects of the Invention

An object of the invention, therefore, is a method for the production of hepatitis B surface antigen in high yields.

Another object of the present invention is the preparation of novel DNA constructs which are capable of expressing hepatitis B surface antigen in high levels.

These and other objects of the invention will become apparent from the disclosure and claims herein provided.

Statement of the Invention

In accordance with the present invention, it has been discovered that hepatitis B surface antigen can be produced in high yields by culturing Pichia cells transformed with DNA constructs comprising hepatitis B surface antigen coding sequences under the control of regulatory regions which are responsive to methanol, non-catabolite repressing carbon sources and carbon source starvation.

Brief Description of the Figures

Figure 1 is a restriction map of the *Pichia* dihydroxyacetone synthase gene (DAS) regulatory region.
Figure 2 is a restriction map of the primary *Pichia* alcohol oxidase gene (AOX1) regulatory region.
Figure 3 is a restriction map of the *Pichia* p40 gene regulatory region.

Figure 4 is a restriction map of plasmid pAOP2.
Figure 5 provides the scheme followed for the construction of plasmids pBSAG5 and pBSAG5I.
Figure 6 is a restriction map of plasmid pHBS-5.
Figure 7 is a restriction map of plasmid pAOT-1.
Figure 8 is a restriction map of plasmid pYJ33.
Figure 9 illustrates the construction of plasmid pYM39 from plasmids pSAOH5 and pTHBS3.
Figure 10 illustrates the construction of plasmid pYMI6 from plasmids pYM39 and pPG3.2.
Figure 11 illustrates the construction of plasmid pBSAGI5I from plasmids pYMI6 and pBSAG5I.
Figure 12 illustrates the insertion of a portion of plasmid pBSAGI5I into the primary alcohol oxidase (AOX1) locus of the *Pichia* chromosome.
Figure 13 provides the scheme followed for the construction of plasmid pTHBS3.
The following abbreviations are used in the Figures to represent the restriction enzymes employed.

Abbreviation	Restriction Enzyme
As	*Asu*II
B	*Bam*HI
B_2	*Bgl*II
Bc	*Bcl*I
C	*Cla*I
H_2	*Hinc*II
H_3	*Hind*III
K	*Kpn*II
Nd_1	*Nde*I
Nr	*Nru*I
Ps	*Pst*I
Pv_1	*Pvu*I
Pv_2	*Pvu*II
R_1	*Eco*RI
R_5	*Eco*RV
S	*Sal*I
Sp	*Sph*I
Ss	*Sst*I
St	*Stu*I
Xb	*Xba*I
Xh	*Xho*I

In the attached figures, restriction sites employed for manipulation of DNA fragments, but which are destroyed upon ligation, are indicated by enclosing the abbreviation for the destroyed site in parenthesis.

Detailed Description of the Invention

In accordance with the present invention, there is provided a novel DNA fragment comprising a regulatory region and a polypeptide coding region wherein the polypeptide coding region codes for hepatitis B surface antigen or portions thereof and the regulatory region is capable of controlling the transcription of messenger RNA when positioned at the 5'-end of the polypeptide-encoding gene. The combination of regulatory region, hepatitis B surface antigen (HBsAg) gene, and a transcriptional terminator fragment is referred to hereinafter as an expression cassette or expression unit. The regulatory region employed in the

practice of the present invention is responsive to at least one of the conditions selected from the group consisting of:

the presence of methanol in the culture medium with which a host organism containing the expression cassette is in contact,

the presence of a non-catabolite repressing carbon source other than methanol in the culture medium with which a host organism containing the expression cassette is in contact, and

carbon source starvation in the culture medium with which a host organism containing the expression cassette is in contact after the host organism has been grown on a catabolite- repressing carbon and energy source.

Further in accordance with the present invention, there are provided novel linear and circular plasmids containing the above described expression cassettes.

Still further in accordance with the present invention, there are provided essentially pure cultures of Pichia strains transformed with the above described linear or circular plasmids.

In accordance with another embodiment of the present invention, process for preparing hepatitis B surface antigen is described which comprises cultivating a Pichia strain transformed with the above described plasmids under conditions where expression of the desired protein product is obtained.

The regulatory regions employed in the practice of the present invention are characterized by their ability to respond to media containing:

(1) methanol,

(2) non-catabolite repressing carbon sources such as, for example, glycerol, galactose, acetate and the like,

(3) catabolite repressing carbon sources, such as, for example, glucose, ethanol, fructose and the like, followed by carbon source starvation.

Exemplary regulatory regions which satisfy the above criteria are depicted by the restriction maps set forth in FIGURES 1, 2 and 3. The regulatory region depicted in FIGURE 1 is derived from the dihydroxyacetone synthase (DAS) gene of *Pichia pastoris*. The regulatory region depicted in FIGURE 2 is derived from the primary alcohol oxidase (AOX1) gene of *Pichia pastoris* (*Pichia* has two alcohol oxidase genes, referred to herein as AOX1 and AOX2). The regulatory region depicted in FIGURE 3 is derived from the p40 gene of *Pichia pastoris*. Those of skill in the art recognize that other regulatory regions having the above described properties can be isolated from methylotrophic yeasts, such as for example, *Pichia pastoris*. Such additional regulatory regions having regulatory properties similar to the properties of the above described regulatory regions are also within contemplation of the present invention.

The hepatitis B surface antigen (HBsAg) gene has been previously isolated (Valenzuela et al. (1979) Nature 280, 815) and is available by appropriate restriction enzyme treatment of a variety of vectors, such as for example, pHBS-5 (see Figure 6, and Valenzuela et al. (1982), Nature 298, 347), pHBV-T-1A (Genentech, EPA 73,657), pHBS-56 (ATCC accession No. 40,047; see EPA 120,551), etc.

The hepatitis B surface antigen gene was modified by Bal31 exonuclease treatment to remove viral noncoding sequences at the 5'- end of the hepatitis gene. The 3'-end of the HBsAg gene was modified by endonuclease digestion and addition of a linker to remove viral noncoding sequences at the 3'-end of the hepatitis gene. The hepatitis B surface antigen gene was further modified to incorporate convenient restriction sites for the manipulation of the DNA fragment. The resulting DNA fragment is an *Eco*RI-*Stu*I insert, and has the following nucleotide sequence:

5'-GAATTCATGG	AGAACATCAC	ATCAGGATTC	CTAGGACCCC
TGCTCGTGTT	ACAGGCGGGG	TTTTTCTTGT	TGACAAGAAT
CCTCACAATA	CCGCAGAGTC	TAGACTCGTG	GTGGACTTCT
CTCAATTTTC	TAGGGGGATC	TCCCGTGTGT	CTTGGCCAAA
ATTCGCAGTC	CCCAACCTCC	AATCACTCAC	CAACCTCCTG
TCCTCCAATT	TGTCCTGGTT	ATCGCTGGAT	GTGTCTGCGG
CGTTTTATCA	TATTCCTCTT	CATCCTGCTG	CTATGCCTCA
TCTTCTTATT	GGTTCTTCTG	GATTATCAAG	GTATGTTGCC
CGTTTGTCCT	CTAATTCCAG	GATCAACAAC	AACCAGTACG
GGACCATGCA	AAACCTGCAC	GACTCCTGCT	CAAGGCAACT
CTATGTTTCC	CTCATGTTGC	TGTACAAAAC	CTACGGATGG
AAATTGCACC	TGTATTCCCA	TCCCATCGTC	CTGGGCTTTC
GCAAAATACC	TATGGGAGTG	GGCCTCAGTC	CGTTTCTCTT
GGCTCAGTTT	ACTAGTGCCA	TTTGTTCAGT	GGTTCGTAGG
GCTTTCCCCC	ACTGTTTGGC	TTTCAGCTAT	ATGGATGATG
TGGTATTGGG	GGCCAAGTCT	GTACAGCATC	GTGAGTCCCT
TTATACCGCT	GTTACCAATT	TTCTTTTGTC	TCTGGGTATA
CATTTAAGGC	CT-3'		

The regulatory region-structural gene constructs of the present invention can be supplied to organisms for amplification, reproduction and expression in a variety of ways. For autonomous replication in yeast, an autonomous replication sequence (ARS) element is useful. Examples include PARS1 and PARS2 derived from *Pichia pastoris* (See copending U.S. Application SN 666,577, Cregg inventor and assigned to Phillips Petroleum Co.). Where integrative transformation of the host is instead desired, no ARS element will be employed. A preferred method to achieve integrative transformation has been described in copending application Serial Number 791,013, by Cregg, assigned to Phillips Petroleum Co., and involves employing a site directed integration vector which comprises

a first insertable DNA fragment,
a selectable marker gene, and
a second insertable DNA fragment.

The first and second insertable DNA fragments are each at least about 200 nucleotides in length and have nucleotide sequences which are homologous to portions of the genomic DNA of species of the genus *Pichia*. The various components of the integrative vector are serially arranged forming a linear fragment of DNA such that the expression cassette and the selectable marker gene are positioned between the 3' end of the first insertable DNA fragment and the 5' end of the second insertable DNA fragment. The first and second insertable DNA fragments are oriented with respect to one another in the serially arranged linear fragment as they are so oriented in the genome of *Pichia*.

It is necessary to include at least one selectable marker gene in the DNA used to transform the host strain. This facilitates selection and isolation of those organisms which have incorporated the transforming DNA. The marker gene confers a phenotypic trait to the transformed organism which the host did not have, e.g., restoration of the ability to produce a specific amino acid where the untransformed host strain has a defect in the specific amino acid biosynthetic pathway.

Those of skill in the art recognize that additional DNA sequences can also be incorporated into the vectors employed in the practice of the present invention, such as for example, bacterial plasmid DNA, bacteriophage DNA, and the like. Such sequences enable the amplification and maintenance of these vectors in bacterial hosts.

Expression in Transformed Yeast

The above-described plasmids of the present invention have utility in Pichia strains which can be transformed. Regulation of gene expression in Pichia by the novel DNA fragments of the present invention can be accomplished by subjecting the transformed organisms to carbon source starvation. Carbon source starvation after growth on a variety of both catabolite repressing and non-catabolite repressing carbon sources induces expression of the gene product maintained under the control of the regulatory regions of the invention. Another means to achieve expression of the desired gene product in appropriate species of transformed Pichia is to grow transformed Pichias on methanol. Yet another means to induce expression of the desired gene product is to grow transformed Pichia on media containing non-catabolite repressing carbon sources.

The regulatory regions of this invention are useful for expression in all Pichia strains, since the regulatory regions have been shown to be induced under a variety of conditions. Thus, Pichias capable of growth on methanol or on non-catabolite repressing carbon sources can be caused to produce foreign, i.e., heterologous, polypeptides directly; while yeasts capable of growth on catabolite repressing carbon sources can be caused to produce foreign polypeptides by subjecting yeast cells so grown to conditions of carbon source starvation.

Pichias are used because their safety of handling, growth conditions and the like have been established and are well known to those of skill in the art.

Pichias are capable of growth on methanol as carbon and energy source.

Since the regulatory regions of the present invention are also induced by growth on non-catabolite repressing carbon sources as well as conditions of carbon source starvation, Pichia strains which are capable of growth on such non-methanolic substrates as:

glucose,
acetate,
glycerol,
ethanol,
lactose,
galactose,
fructose,
sucrose,

and the like and mixtures of any two or more thereof are also useful in the practice of the invention. By growing the host organism on a suitable non-catabolite repressable, non-methanolic carbon source such as, for example, glycerol or galactose, or by growing the host organism on a suitable catabolite repressable carbon source such as, for example, ethanol, glucose and fructose, then subjecting the host organism to carbon source starvation conditions, expression of a gene product under the control of the regulatory regions of the invention can be achieved.

An especially preferred host Pichia strain is the mutant *Pichia pastoris* GS115, which is a mutant defective in the ability to produce histidine. GS115 has been designated as having the mutant genotype *his4*, as a result of the defect in the histidine pathway affecting the histidinol dehydrogenase-encoding gene. GS115 is derived from *Pichia pastoris* NRRL Y-11430 and has been deposited with the Northern Regional Research Center of the United States Department of Agriculture in Peoria, Illinois, and has been assigned the accession number NRRL Y-15851. This particular host is useful because it is an auxotrophic mutant deficient in the histidine pathway. Transformation of this host with a vector containing, among other DNA sequences, sequences encoding the HIS4 gene function, allows ready selection of transformed hosts.

Another preferred Pichia strain for use in the practice of the present invention is the mutant *Pichia pastoris* GS190, which is a mutant defective in the arginine pathway affecting the argininosuccinate lyase encoding gene. GS190 is derived from *Pichia pastoris* NRRL Y-11430, and has been deposited with the Northern Regional Research Center of the United States Department of Agriculture in Peoria, Illinois, and has been assigned the accession number NRRL Y-18014.

Yet another preferred host Pichia strain is the double auxotrophic mutant PPF1, which is a mutant defective in both the histidine and arginine pathways. PPF1 is defective in both the histidine pathway affecting the histidinol dehydrogenase encoding gene and the arginine pathway affecting the argininosuccinate lyase encoding gene. PPF1 has been deposited with the Northern Regional Research Center of the United States Department of Agriculture in Peoria, Illinois, and has been assigned the accession number NRRL Y-18017.

Escherichia coli is also a suitable host for the plasmids of the invention. Those of skill in the art recognize that many strains of *E. coli* are suitable hosts. Several strains employed in the present work are

summarized below:

Strain designation	Accession Number
MC1061	None known
LE392	ATCC #33572
MM294	ATCC #33625

Pichia pastoris Transformation Procedure

The experimental procedures for the transformation of *Pichia pastoris* have been previously described, and are presented in greater detail below (Example I).

Pichia pastoris can be transformed by enzymatic digestion of the cell walls to give spheroplasts; the spheroplasts are then mixed with the transforming DNA and incubated in the presence of calcium ions and polyethylene glycol, then regenerated in selective growth medium deficient in histidine. The transforming DNA includes the HIS4 gene in which the host strain is deficient, thus only transformed cells survive on the selective growth medium employed.

Hepatitis B Surface Antigen Extraction

Those of skill in the art are aware of numerous methods available for the extraction of a heterologous protein from a unicellular recombinant host. Any of the techniques known by those of skill in the art for cell disruption and protein concentration and/or extraction from the disrupted cells are suitable for recovery of the HBsAg produced in accordance with the present invention.

Hepatitis B Surface Antigen Assays

The transformed cells were grown under appropriate conditions for expression, as described above. Then, following cell breakage, the soluble and insoluble fractions were analyzed for HBsAg. The soluble fraction was analyzed for 22nm particle with a commercially available "AUSRIA® II" analysis kit (Abbott Laboratories). Both the soluble and insoluble fractions were analyzed for monomer by employing Western blotting procedures which used antisera raised against the monomeric form of HBsAg and radioactive ^{125}I-labeled protein A.

The invention will now be described in greater detail with reference to the following non-limiting examples.

EXAMPLES

The following abbreviations are used throughout the examples, with the following meaning:

SDS	sodium dodecylsulfate
EDTA	ethylenediamine tetraacetic acid
TEMED	N,N,N′,N′-tetramethylenediamine
DTT	dithiothreitol
BSA	bovine serum albumin
EtBr	ethidium bromide
PMSF	phenylmethylsulfonyl fluoride
Ci	Curie

Zymolyase 60,000 Source: Miles Laboratories

The buffers and solutions employed in the following examples have the compositions given below:

1M Tris buffer	121.1 g Tris base in 800 mL of H_2O; adjust pH to the desired value by adding concentrated (35%) aqueous HC1; allow solution to cool to room temperature before final pH adjustment, dilute to a final volume of 1L.

TE buffer	1.0 mM EDTA
	in 0.01 M (pH 7.4) Tris buffer
PBS (Phosphate buffered saline)	10 mM sodium phosphate (pH 7.0)
	0.15 M NaCl
SDS Gel Loading Buffer	62.5 mM Tris-HCl (pH 6.8)
	2% SDS
	10% glycerol
	100 mM dithiothreitol
	0.001% bromphenol blue
YPD Medium	1% Bacto-yeast extract
	2% Bacto-peptone
	2% Dextrose
SD Medium	6.75 g yeast nitrogen base without amino acids (DIFCO)
	2% Dextrose
	in 1 L of water
SED	1 M Sorbitol
	25 mM EDTA
	50 mM DTT
SCE Buffer	9.1 g Sorbitol
	1.47 g Sodium citrate
	0.168 g EDTA
	50 mL H_2O
	--pH to 5.8 with HCl
CaS	1 M Sorbitol
	10 mM $CaCl_2$
	--filter sterilize
PEG Solution	20% polyethylene glycol-3350
	10mM $CaCl_2$
	10mM Tris-HCl (pH 7.4)
	--filter sterilize
SOS	1 M Sorbitol
	0.3x YPD medium
	10 mM $CaCl_2$

Basal Salt Composition (for fermentor growth of transformed *Pichia*)

Basal Salts	per liter
H_3PO_4, 85%	4.2 mL
$CaSO_4 \cdot 2H_2O$	0.18 gm
K_2SO_4	2.86 gm
$MgSO_4 \cdot 7H_2O$	2.34 gm
KOH	0.65 gm

Pichia Feed Medium (for fermentor growth of GS115/pBSAG5)	
	g/L Water
H_3PO_4 (85%)	3.5 mL
$CaSO_4 \cdot 2H_2O$	0.15
K_2SO_4	2.38
$MgSO_4 \cdot 7H_2O$	1.95
KOH	0.65
$FeSO_4 \cdot 7H_2O$	0.065
$CuSO_4 \cdot 5H_2O$	0.006
$ZnSO_4 \cdot 7H_2O$	0.020
$MnSO_4 \cdot H_2O$	0.003
Biotin	0.000041
Carbon Source	20-100 g

Trace Salts Solution [for growth of GS115(pBSAGI5I)]	
	g/L Water
$CuSO_4 \cdot 5H_2O$	0.06
KI	0.08
$MnSO_4 \cdot H_2O$	0.3
$Na_2MoO_4 \cdot 2H_2O$	0.2
H_3BO_3	0.02
$ZnSO_4 \cdot 7H_2O$	2.0
$FeCl_3 \cdot 6H_2O$	4.8
H_2SO_4	3-5 mL/L (to remove cloudiness)

Ausria dilution buffer	4.3 mM Na_2HPO_4 1.5 mM KH_2PO_4 2.7 mM KCl 0.15 M NaCl 1% bovine serum albumin 0.02% sodium azide --final pH 7.4
Solubilization buffer	10 mM Sodium phosphate buffer (pH 7.5) 0.5 M NaCl 0.1% Triton X-100 2 mM PMSF

Unless otherwise specified, the above solutions represent the basic (1x) concentration employed. Throughout the examples, where the different concentration levels are employed, that fact is indicated by referring to the solution as a multiple of the basic (1x) concentration.

EXAMPLE I

Pichia pastoris Transformation Procedure

A. Cell Growth

1. Inoculate a colony of *Pichia pastoris* GS115 (NRRL Y-15851) into about 10 mL of YPD medium and shake culture at 30°C for 12-20 hrs.
2. After about 12-20 hrs., dilute cells to an OD_{600} of about 0.01-0.1 and maintain cells in log growth phase in YPD medium at 30°C for about 6-8 hrs.

3. After about 6-8 hrs, inoculate 100 mL of YPD medium with 0.5 mL of the seed culture at an OD_{600} of about 0.1 (or equivalent amount). Shake at 30°C for about 12-20 hrs.
4. Harvest culture when OD_{600} is about 0.2-0.3 (after approximately 16-20 hrs) by centrifugation at 1500 g for 5 minutes.

B. Preparation of Spheroplasts

1. Wash cells once in 10 mL of sterile water. (All centrifugations for steps 1-5 are at 1500 g for 5 minutes.)
2. Wash cells once in 10 mL of freshly prepared SED.
3. Wash cells twice in 10 mL of sterile 1 M Sorbitol.
4. Resuspend cells in 10 mL SCE buffer.
5. Add 5-10 µL of 4 mg/mL Zymolyase 60,000 (Miles Laboratories). Incubate cells at 30°C for about 30-60 minutes.

Since the preparation of spheroplasts is a critical step in the transformation procedure, one should monitor spheroplast formation as follows: add 100 µL aliquots of cells to 900 µL of 5% SDS and 900 µL of 1 M Sorbitol before or just after the addition of zymolyase and at various times during the incubation period. Stop the incubation at the point where cells lyse in SDS but not in sorbitol (usually between 30 and 60 minutes of incubation).

6. Wash spheroplasts twice in 10 mL of sterile 1 M Sorbitol by centrifugation at 1000 g for 5-10 minutes. (The time and speed for centrifugation may vary; centrifuge enough to pellet spheroplasts but not so much that they rupture from the force.)
7. Wash cells once in 10 mL of sterile CaS.
8. Resuspend cells in total of 0.6 mL of CaS.

C. Transformation

1. Add DNA samples (up to 20 µL volume) to 12 × 75 mm sterile polypropylene tubes. (DNA should be in water or TE buffer; for maximum transformation frequencies with small amounts of DNA, it is advisable to add about 1 µL of 5 mg/mL sonicated *E. coli* DNA to each sample.)
2. Add 100 µL of spheroplasts to each DNA sample and incubate at room temperature for about 20 minutes.
3. Add 1 mL of PEG solution to each sample and incubate at room temperature for about 15 minutes.
4. Centrifuge samples at 1000 g for 5-10 minutes and decant PEG solution.
5. Resuspend samples in 150 µL of SOS and incubate for 30 minutes at room temperature.
6. Add 850 µL of sterile 1 M Sorbitol and plate aliquots of samples as described below.

D. Regeneration of Spheroplasts

1. Recipe for Regeneration Agar Medium:
   a. Agar-KCl- 9 g Bacto-agar, 13.4 g KCl, 240 mL H_2O, autoclave.
   b. 10× Glucose- 20 g Dextrose, 100 mL H_2O, autoclave.
   c. 10× SC- 6.75 g Yeast Nitrogen Base without amino acids, 100 mL H_2O, autoclave. (Add any desired amino acid or nucleic acid up to a concentration of 200 µg/mL before or after autoclaving.)
   d. Add 30 mL of 10× Glucose and 30 mL of 10× SC to 300 mL of the melted Agar-KCl solution. Add 0.6 mL of 0.2 mg/mL biotin and any other desired amino acid or nucleic acid to a concentration of 20 µg/mL. Hold melted Regeneration Agar at 55-60°C.
2. Plating of Transformation Samples:

Pour bottom agar layer of 10 mL Regeneration Agar per plate at least 30 minutes before transformation samples are ready. Distribute 10 mL aliquots of Regeneration Agar to tubes in a 45-50°C bath during the period that transformation samples are in SOS. Add a quantity of each sample to 10 mL aliquots of melted Regeneration Agar held at 45-50°C and pour each onto plates containing a solid 10 mL bottom agar layer of Regenation Agar.

3. Determination of Quality of Spheroplast Preparation:

Remove 10 µL of one sample and dilute 100 times by addition to 990 µL of 1 M Sorbitol. Remove 10 µL of the 100 fold dilution and dilute an additional 100 times by addition to a second 990 µL aliquot of 1 M Sorbitol. Spread plate 100 µL aliquots of both dilutions on YPD agar medium to determine the concentration of unspheroplasted whole cells remaining in the preparation. Add 100 µL of each dilution

to 10 mL of Regeneration Agar supplemented with 40 µg/mL histidine to determine total regeneratable spheroplasts. Good values for a transformation experiment are 1-3 X 10^7 total regeneratable spheroplasts/mL and about 1 X 10^3 whole cells/mL.
4. Incubate plates at 30° C for 3-5 days.

EXAMPLE II

Construction of the pAOP2 Family of Vectors

1. Plasmid pPG2.5 (a pBR322 based plasmid containing the approximately 2.5 Kbp *Eco*RI-*Sal*I fragment from plasmid pPG4.0, which plasmid contains the primary alcohol oxidase gene (AOX1) and regulatory regions and which is available in an *E. coli* host from the Northern Regional Research Center of the United States Department of Agriculture in Peoria, Illinois as NRRL B-15868) was digested with *Bam*HI.
2. The linearized plasmid was digested with *BAL*31;
3. The resulting DNA was treated with Klenow fragment to enhance blunt ends, and ligated to *Eco*RI linkers;
4. The ligation products were transformed into *E. coli* strain MM294;
5. Transformants were screened by the colony hybridization technique using a synthetic oligonucleotide having the following sequence:
5'TTATTCGAAACGGGAATTCC.
This oligonucleotide contains the AOX1 promoter sequence up to, but not including, the ATG initiation codon, fused to the sequence of the *Eco*RI linker;
6. Positive clones were sequenced by the Maxam-Gilbert technique. All three positives had the following sequence:
5'...TTATTCGAAACGAGGAATTCC...3'.
They all retained the "A" of the ATG (underlined in the above sequence). It was decided that this A would probably not be detrimental; thus all subsequent clones are derivatives of these positive clones. These clones have been given the laboratory designation pAOP1, pAOP2 and pAOP3, respectively.
7. Two other clones were identified by screening of the *BAL*31/linker-ligated products. They have the following sequence:

$$5'\ldots\underbrace{\mathrm{T\ A\ A\ T\ T\ A\ T\ T\ C\ G}}_{\mathrm{pAOX1}}\ \underbrace{\mathrm{G\ A\ A\ T\ T\ C}}_{Eco\mathrm{RI}}\ \mathrm{C}\ldots 3'$$

These clones have been designated pAOP5 and pAOP6.

In a variation of the above procedure, plasmid pPG2.5 was cut with *Asu*II, instead of *Bam*HI, the linearized fragment was treated with Klenow fragment (no *BAL*31 treatment as done above), then ligated to *Eco*RI linkers. The resulting plasmid contains AOX1 promoter sequences, absent the ATG initiation codon. The plasmid thus prepared has been designated pAOP4, and has the following sequence:

$$5'\ldots\underbrace{\mathrm{T\ A\ A\ T\ T\ A\ T\ G}}_{\mathrm{pAOX1}}\ \underbrace{\mathrm{G\ A\ A\ T\ T\ C}}_{Eco\mathrm{RI}}\ldots 3'$$

The AOX1 promoter (pAOX1) responds to carbon catabolic repression by a severe cessation of enzyme synthesis. In addition, the AO promoter responds to carbon starvation. Growth on methanol leads to a further induction of the AOX1 promoter. Furthermore, it is clear from extensive studies, such as those described by Ellis, Brust, Koutz, Waters, Harpold and Gingeras in Molecular and Cellular Biology, May, 1985, p. 1111-1121, that the AOX1 promoter fragment used in this invention is regulated in a similar fashion to the AOX1 promoter in the chromosome. Each of the clones prepared and isolated as described in this example display responses to catabolic repression, carbon starvation and methanol induction as does the AOX1 promoter itself.

Description of the AO Terminator

The *StuI-HindIII* fragment used as the AO terminator contains sequences which provide a template for polyadenylation of the AOX1 mRNA transcript. These sequences include the following:

TATAGTATAGGATTTTTTTTTGTC-polyadenylation.

When the *StuI-HindIII* fragment is located on a plasmid 3' to a polypeptide coding region, it promotes RNA termination. The AOX1 termination sequences have been isolated, and can be recovered from plasmid pPG3.2, which is a pBR322-based plasmid containing the AOX1 termination sequences. The plasmid, transformed into an *E. coli* host, is available to the public from the Northern Regional Research Center of the United States Department of Agriculture in Peoria, Illinois under the accession number NRRL B-15999.

EXAMPLE III

The sequence of steps employed for the preparation of the plasmids which are the subject of this example are summarized in attached Figure 5.

Construction of pBSAG5 and pBSAG5I

1. Construction of pCFL2

Vector pAOP2, which contains the AOX1 promoter minus the TG of the ATG at its 3'-end, was cut with *HincII*. The promoter-containing DNA fragment was isolated and ligated into pBR322 which was previously cut with *HindIII* and filled in with Klenow fragment. This reaction created vector pCFL2.

2. Construction of pBSAOP2

pBR322-*BglII*, which is pBR322 with the *PvuII* site replaced by a *BglII* site, was digested with *EcoRI* and *ClaI*. This linearized plasmid was combined with the 5' AOX1-containing *ClaI/EcoRI* fragment from pCFL2 in a ligation reaction. The resulting vector was designated pBSAOP2.

3. Construction of pBSAG22

Plasmid pHBS-5 (described by Valenzuela et al. in Nature 298, 347-350 (1982); see Figure 6), which contains the HBsAg gene inserted into the *EcoRI* site in pBR322, was digested with *ClaI*. Approximately 60 base pairs were removed in both directions with *Bal31* exonuclease. The remaining DNA fragment was digested with *BamHI* and filled in with Klenow fragment. After ligation, a pool of approximately 200 transformants were cut with *NcoI*. The linearized plasmids were isolated and religated. After transformation of *E. coli*, approximately 10% of all plasmids (designated pBSAG1) had a newly created *NcoI* site. pBSAG1 was digested with *NcoI*, filled in with Klenow fragment and digested with *BamHI*. This plasmid fragment was ligated to pBSAOP2, which was previously digested with *EcoRI*, filled in with Klenow fragment and digested with *BamHI*. The resulting vector was designated pBSAG22.

4. Construction of pBSAG4, pBSAG5, pBSAG5I

Plasmid pAOT-1, a pBR322 based plasmid derived by ligating the 1.6 kbp *SalI-HindIII* fragment of pPG3.2 (available in an *E. coli* host as NRRL B-15999) into a *SalI-HindIII* cut pBR322 Δ *EcoRI* (i.e., pBR322 with the *EcoRI* site destroyed; see Figure 7), which carries the 3'-AOX1 transcriptional termination fragment, was cut with *XbaI* and *PstI*. The terminator-containing fragment was ligated to pBSAG22, which was previously cut with *XbaI* and *PstI*, yielding pXP-1. pBSAG22 was digested with *DraI*, then *StuI* linkers were added, and finally *StuI* and *EcoRI* were used for further digestion. The HBsAg structural gene was isolated and ligated into pXP-1, which had been previously cut with *StuI* and *EcoRI*, yielding pBSAG4.

The HBsAg containing *ClaI* fragment from pBSAG4 was ligated into the unique *ClaI* site of pYJ33 (see Figure 8) yielding pBSAG5 and pBSAG5I. A restriction map of plasmid pBSAG5I is presented in Figure 11. Plasmids pBSAG5 and pBSAG5I differ only in the orientation of the *ClaI* fragment which contains the 5'-AOX1/HBsAg/3'-AOX1 expression cassette. Thus, in pBSAG5, the 5'-AOX1 fragment is adjacent to the *Pichia* HIS4 gene, while the 3'-AOX1 fragment is adjacent to the autonomous element, PARS2. Plasmid pBSAG5, transformed into an *E. coli* host, has been deposited with the Northern Regional Research Center of the United States Department of Agriculture The *E. coli* strain MC1061-pBSAG5 has been assigned the accession number NRRL B-18028.

EXAMPLE IV

CONSTRUCTION OF THE *PICHIA PASTORIS* HBsAg EXPRESSION HOST GS115 (pBSAGI5I)

The preparation of a *Pichia pastoris* host in which the primary alcohol oxidase gene (AOX1) is replaced by the Hepatitis B surface antigen (HBsAg) gene in the *Pichia* chromosome is described in this Example.

To produce the *P. pastoris* HBsAg expression-Aox1⁻ mutant host, plasmid pBSAGI5I was constructed as outlined in Figures 9-11. The first step in the construction was to digest the AOX1 promoter-LacZ gene expression vector pSAOH5 and the AOX1 promoter-HBsAg expression vector pTHBS3, prepared as described below and summarized in Figure 13, with restriction endonuclease *Hin*dIII. To prepare pTHBS3, plasmid pAOT-1 (see Figure 7) was cut with *Stu*I, ligated with *Eco*RI linkers, and then digested with *Pst*I. The *Eco*RI- *Pst*I fragment containing the 3'-AOX1 fragment was isolated. Vector pAOP3, which contains 5'-AOX1 sequences, was cut with *Eco*RI and *Sst*I; the resulting 5'-AOX1 fragment was ligated into the *E. coli–S. cerevisiae* shuttle vector pSEY101 (Douglas et al. (1984) Proc. Natl. Acad. Sci. USA, 81, 3983-3987) which had previously been cut with *Eco*RI and *Sst*I. The result of ligating these pAOP3 and pSEY101 fragments was plasmid pTAO20. Plasmid pTAO20 contains the URA3 and ampicillin genes for selection in *S. cerevisiae* and bacteria, respectively, the 2μ circle for replication in *S. cerevisiae*, and the 5'-AOX1 sequences.

Plasmid pTAO20 was partially cut with *Pst*I. The linearized vector was isolated and cut with *Eco*RI. The largest fragment (which contained the 2μ circle sequences, the URA3 gene and the 5'-AOX1 fragment) was ligated to the 3'-AOX1 fragment obtained from pAOT-1, to produce vector pTHBS1.

The HBsAg-containing *Eco*RI fragment from pHBS-5 isolated by digestion with *Eco*RI, then ligated with pTHBS1, which had previously been digested with *Eco*RI and treated with bacterial alkaline phosphatase. The resulting vector, designated pTHBS2, has the HBsAg gene inserted between the 3'-and 5'-AOX1 sequences.

Plasmid pYJ30 (available in an *E. coli* host as NRRL B-15890) was cut with *Eco*RI, filled in with Klenow fragment, then cut with *Pst*I. The *P. pastoris* HIS4/PARS1-containing fragment was isolated and ligated with the *Pst*I-*Sst*I fragment from vector pTHBS2 (which contains the HBsAg gene flanked by the AOX1 sequences). This ligation yields vector pTHBS3.

The 1.4 kbp fragment obtained from pTHBS3 upon digestion with *Hin*dIII (which fragment contains the HBsAg gene, the AOX1 termination sequence and a portion of the AOX1 promoter sequence) was recovered and inserted into the 7.7 kbp fragment from pSAOH5, which contains the *Pichia* HIS4 gene, most of the AOX1 promoter sequence, and sequences from pBR322. A 9.1 kbp recombinant plasmid, pYM39, which contains the restored AOX1 promoter sequences, was then isolated.

For the second construction step, the plasmid pPG3.2 (available in an *E. coli* host as NRRL B-15999) was digested with *Pvu*II and a 1.5 kbp fragment which contains sequences immediately 3' of the AOX1 gene was inserted into the single *Nru*I site of pYM39. A 10.6 kbp recombinant plasmid, pYMI6, was isolated which contained the *Pvu*II fragment oriented such that the 3' AOX1 gene proximal sequences were oriented toward the HIS4 gene portion of the vector. Plasmid pYMI6 contained all components required for deletion of the AOX1 gene from a *Pichia* host, and expression of HBsAg with AOX1 promoter control, but it does not contain the trimmed HBsAg gene fragment of pBSAG5.

Therefore, the last construction step was to recombine the desired HBsAg gene into an AOX1 gene deletion vector. For this, pYMI6 and pBSAG5I, (a plasmid identical to pBSAG5 except that the *Cla*I fragment which contains the HBsAg expression cassette is in the opposite orientation) were digested with restriction enzymes *Pst*I and *Sph*I. The 6.3 kbp fragment from pBSAG5I, which contains the trimmed HBsAg gene expression cassette and the *Pichia* HIS4 gene, was inserted into the 4.6 kbp fragment from pYMI6 which contains the 3' AOX1 sequences and most of pBR322 to produce the final 10.9 kbp plasmid, pBSAGI5I. Plasmid pBSAGI5I, carried in an *E. coli* host, has been deposited with the Northern Regional Research Center in Peoria, Illinois and has been assigned acession number NRRL B-18021.

To transform the *P. pastoris his4* mutant strain GS115 (NRRL Y-15851), pBSAGI5I was first digested with restriction enzyme *Bgl*II to produce a 7.2 kbp linear vector, which contains 0.85 kbp of sequence from 5' of the AOX1 gene at one terminus and 1.1 kbp of sequence from 3' of the AOX1 gene at the other terminus (Figure 12). About 2 μg of *Bgl*II-cut pBSAGI5I was transformed into GS115 by selection for histidine prototrophy. Approximately 5 x 10^3 His^+ colonies resulted from the transformation.

Transformation events in which pBSAGI5I was inserted as a linear molecule at the AOX1 chromosomal locus result in the deletion of the AOX1 gene. Therefore, His^+-transformed strains in which the desired linear insertion had occurred were identified by their very slow growth rate on methanol. (*P. pastoris* has a second "weaker" alcohol oxidase gene, AOX2, which produces alcohol oxidase sufficient for methanol

growth at a slow rate in strains defective in the primary alcohol oxidase gene.)

The procedure for identifying the His+ transformants which could not grow well on methanol was to first recover the His+ cells which were embedded in the selective agar. The recovery step was performed by transferring the agar to a 50 mL tube containing 20 mL of sterile water and pulverizing the agar using a Brinkman homogenizer at low speed for 30 seconds. Agar debris was separated from the cells by filtering the mixture through gauze and rinsing the agar with 30 mL of sterile water. The yeast cells were then diluted to an optical density at A_{600} of 0.1, sonicated for 10 seconds using a Branson sonifier at setting 4 to break apart yeast cell clumps and diluted one hundred fold with sterile water. Aliquots of 10 and 100 µL were spread on agar plates containing 0.67% yeast nitrogen base without amino acids (Difco) and 0.1% glucose. After incubation at 30 °C for 3 days, colonies which appeared on the plates were screened for the ability to grow on methanol by replica plating the colonies onto a series of agar plates containing 0.67% yeast nitrogen base without amino acids and the following carbon sources: 1) no carbon source; 2) 0.5% methanol; and 3) 2% glucose. Of the colonies which grew on 2% glucose, 32% could not grow well on methanol.

To confirm that the pBSAGI5I sequences were inserted as shown in Figure 12, total DNA was extracted from one of the *P. pastoris* strains defective in methanol utilization, digested with restriction endonucleases and hydridized by the Southern blot method with ^{32}P-labelled probes. In one set of Southern blots, DNAs from the Aox1$^-$ strain, GS115(pBSAGI5I), and the Aox1$^+$ straining GS115, were digested with *Hin*dIII and hybridized with labeled pPG4.0, a plasmid composed of the AOX1 gene and sequences from pBR322 available in an *E. coli* host from the Northern Regional Research Center in Peoria, Illinois as NRRL B-15868. A 2.3 kbp fragment which encodes AOX1 was seen in the lanes containing GS115 DNA. However, the 2.3 kbp fragment was absent and no new fragments appeared in lanes which contained GS115(pBSAGI5I) DNA. This result demonstrated that the AOX1 gene had been deleted from the GS115(pBSAGI5I) strain.

EXAMPLE V

GROWTH OF *PICHIA* YEASTS TRANSFORMED WITH HBsAg-ENCODING VECTORS

1. Growth of GS115(pBSAG5) in a fermentor

A 10% inoculum was grown overnight in yeast nitrogen base (YNB) + 2% glucose in a shake flask at 30 °C. The inoculum was added to sterilized basal salts (adjusted to pH 4) in the fermentor. Glucose feed was added at a dilution rate of 0.05 to 0.1 h^{-1}. When cell density reached a steady state level and fermentor glucose levels approached less than 100 ppm, HBsAg production was induced by changing the feed carbon source to methanol or a 50% glucose-50% methanol mixture.

2. Growth of GS115 (pBSAGI5I) (Aox1$^-$) in a fermentor

Optimum expression of soluble HBsAg Ausria activity (3-4% of soluble protein) has been achieved by growing this Aox1$^-$ organism in a batch mode on glycerol, followed by a methanol-containing feed. Inoculum can be grown on YNB + glycerol. Basal salts plus glycerol (1% and 4% glycerol have been used) and biotin can be autoclaved in the fermentor. After cooling, the pH should be adjusted to be between 3.5 and 6, and trace salts (2.5 mL/L) added before inoculating. One hundred percent methanol can be started before or after the glycerol has been exhausted. Methanol levels as high as 2% do not interfere with HBsAg accumulation, which can continue as for long as 200 hours. However, 5% methanol in the fermentor will stop the accumulation of HBsAg particle.

Growth to higher cell densities has been achieved by increasing the feed salt concentrations. Increased zinc levels are particularly important for increased cell densities when grown on methanol. Higher levels of extractable Ausria activity have been achieved when growth was not limited by zinc, but the extractable protein was also higher, resulting in a net decrease in Ausria activity as a percent of soluble protein.

3. Shake Flask Growth of Cell Cultures of GS115(pBSAG5) and GS115(pBSAGI5I)

A transformed colony was picked and streaked out on a SD plate. A streak of cells was inoculated in 50 mL of YNB broth (1 × YNB, 5 µg/mL of biotin) with 5% glucose in a 250 mL shake flask, and shaken at 30 °C at 250 revolutions per minute in an airshaker overnight. The morning OD_{600} reading was between 2-3. 100 OD_{600} units of cells (about 10^9 cells) were removed from the shake flask and centrifuged in an IEC centrifuge for 7 minutes at 2000 Xg at room temperature. The cell pellet was resuspended in 500 mL YNB

broth with 2% glycerol in a 2 liter shake flask (OD_{600} = 0.2). The culture was incubated at 30 °C and 250 rpm in an airshaker until the OD_{600} reached 2 to 3 OD_{600}. In the case of the Aox1^{-} host, 500 OD_{600} were removed from the culture. The cell suspension was centrifuged in an IEC for 7 minutes at 2000 Xg. The cell pellet was resuspended in 500 mL YNB broth with 0.5% methanol (1.0 OD_{600}). In the case of the Aox1^{+} host, 170 OD_{600} of cells were removed from the culture, centrifuged under the same conditions and resuspended in 500 mL YNB broth with 0.5% methanol (0.3 OD_{600}). Both cultures were shaken in 2 liter shake flasks at 30 °C and 250 rpm. Whenever an $OD_{600} \geq 2$ was obtained, the cultures were diluted two-fold with the same growth media. 100 OD_{600} samples were removed periodically and centrifuged for 7 min at 2000 Xg. The resulting cell pellets can be stored frozen at -70 °C for 1 to 2 weeks.

EXAMPLE VI

ASSAYS OF HBsAg: 22nm PARTICLE AND HBsAg MONOMER

1. Preparation of Extracts and Protein Determination

All of the following operations were performed at 0-4 °C. The frozen cell pellet was thawed, then washed twice with 2 mL of ice cold Solubilization buffer. The cells (100 OD_{600} units) were transferred into a disposable glass tube (13x 100 mm). 0.35 mL of Solubilization buffer and 0.5 g of acid-washed glass beads (0.45 mm in diameter) were added to the cell pellet. This suspension was shaken on a vortex mixer on maximum setting 4 times for 1 minute each, and held for 1 minute intervals between each shaking on ice. The whole cell slurry was removed and the glass beads were washed with 0.35 mL Solubilization buffer. The wash buffer was combined with the cell slurry and transferred into an Eppendorf tube. The extract was centrifuged in an Eppendorf centrifuge for 15 minutes. The supernatant (soluble fraction; 0.7 mL) was removed from the pellet. To extract HBsAg protein from the pellet, 0.7 mL of 2x concentrated SDS-Solubilization buffer was added to the pellet, and the mixture was stirred on a vortex mixer and boiled for 15 minutes. The mixture was centrifuged for 15 minutes, then the supernatant (insoluble fraction) was removed from the cell debris. Aliquots from the soluble and insoluble fractions were assayed for protein content using the TCA precipitation and the Lowry method. BSA served as a protein concentration standard. Both the insoluble and soluble fractions usually had protein concentrations in the range of 3-15 mg/mL.

2. Alternate Procedure for Preparation of Extracts

This protocol describes conditions for extraction of the monomeric heterologous protein HBsAg, or the protein complex, 22 nm particle, from cultures of *Pichia pastoris* transformed with vectors containing sequences that code for the HBsAg protein.

Cultures of *P. pastoris* were grown to a cell density of 10-100 OD_{600} units per milliliter. An aliquot of 100 OD_{600} units was transferred to a 13 x 100 mm borosilicate culture tube and washed twice with 20 volumes of solubilization buffer.

The cells were pelleted, then to the pelleted cells (IEC clinical centrifuge) was added 0.5 g of acid-washed glass beads (0.5 mm) followed by 0.35 mL of solubilization buffer. The solubilization buffer contained either 0.5 $\underline{M}$ NaCl and 0.1% Triton X-100 (wt/vol.) as a control, or a 3 $\underline{M}$ concentration of potassium iodide or potassium thiocyanate, in the presence or absence of 0.1% Triton X-100. All solutions were buffered at pH = 7.5 with 10 mM sodium phosphate. The mixture was agitated for four, one-minute intervals at maximum speed using a vortex mixer. Between intervals the mixture was cooled on ice for not less than one minute. After lysing was completed, the solution of broken cells was removed, the glass beads were washed with 0.35 mL of solubilization buffer, and the two solutions were combined and subjected to centrifugation for 15 minutes at 13,000 xg. The supernatants were removed and assayed for immunoreactive HBsAg particle (Ausria assay) and total trichloroacetic acid precipitable protein (Lowry). The results, as a range of 5 experiments, are presented in Table I.

TABLE I

Lysing Conditions	A HBsAg 22nm Particle (μg/mL)	B Total Protein (μL/mL)	C HBsAg 22 nm Particle/Protein (wt %)
Salt (conc.)			
NaCl (0.5 M) + Triton	203-249	8.6-11.2	2.1-3.2
KI (3M) + Triton	5.1-150	0.85-3.4	0.5-8.1
KI (3M) - Triton	71-136	2.5-4.3	2.3-7.2
KSCN (3M) + Triton	2.4-50	0.6-1.9	0.8-9.6
KSCN (3M) - Triton	80-125	1.6-4.3	3.8-16.7

While none of the conditions containing potassium iodide or potassium thiocyanate, yields values for HBsAg particle higher than the conditions employing sodium chloride (column A), it is clear that potassium iodide or potassium thiocyanate, inhibit the release of total protein (column B), thereby increasing the specific activity of the particle 2-5 fold (column C).

3. 22 nm Particle Assays (AUSRIA™ II kit)

The soluble fraction was diluted 1000 to 10,000-fold with Ausria dilution buffer, and aliquots between 25 and 100 μL were assayed as follows:

First Incubation

1. To construct a standard curve, a dilution series containing between 0.1 ng up to 4 ng of control in a total volume of 200 μL each was pipetted into the bottom of individual wells of a reaction tray (along with 4 negative controls).

For the samples to be analyzed, 200 μL of each diluted soluble fraction was pipetted into the bottom of separate wells of the reaction tray.

2. One bead was carefully added to each well containing a sample fraction or control. Alternatively, beads may be dispensed prior to the addition of controls or samples.

3. The cover seal was applied to the reaction tray, which was then gently tapped to cover the beads and to remove any trapped air bubbles.

4. The reaction was then incubated at 45 °C for 2 hours.

5. The cover seal was removed and discarded. The liquid was aspirated, and each bead washed two times with 4 to 6 mL of distilled or deionized water.

Second Incubation

6. 200 μL of ^{125}I-Anti-HBs was pipetted into each well containing a bead.

7. A new cover seal was applied, and the reaction tray gently tapped to cover the beads and to remove any trapped air bubbles.

8. The reaction tray was then incubated at 45 °C for 1 hour.

9. The cover seal was removed and discarded. The liquid was aspirated and each bead washed four times as in step 5.

10. The beads were then immediately transferred to properly identified counting tubes.

Gamma Scintillation Counter Reading:

11. The count rate was determined for one minute.

12. The samples were counted within 24 hours after the final wash.

The level of 22 nm particles was calculated using the standard curve generated as described in step 1.

4. Monomer Assay (Western Assay)

The equivalent volume of 25 µg of protein (soluble or insoluble fraction), usually 2-5 µL, was brought up with H_2O to 10 µL. 10 µL of 2x concentrated SDS gel loading buffer (100 mM DTT in 1x buffer) was added and the sample was boiled for 15 min. The boiled samples were loaded on a 12% SDS acrylamide gel (Laemmli). After gel electrophoresis, the proteins were transferred to nitrocellulose paper (Towbin *et al.* Proc. Natl. Acad. Sci. USA 76, 4350-4354 (1979)). The HBsAg was detected with HBsAg antisera (raised against plasma-derived HBsAg) and ^{125}I-labelled protein A. The nitrocellulose paper was exposed to Kodak XAR-5 film overnight at -70 °C. Quantitation of monomer was done by counting the radioactive bands from the nitrocellulose paper in a gamma-counter. Recombinant HBsAg produced by *S. cerevisiae* (100-500 ng/lane) was used as a standard.

EXAMPLE VII

Expression Levels of HBsAg in *Pichia pastoris*

The production of HBsAg by several transformed *P. pastoris* strains was determined by the assay protocol set forth in Example VI, using the solubilization protocol described in part 1 of Example VI. Results are summarized in Table II.

TABLE II

transformed strain	GS115 (pBSAGI5I)	GS115 (pBSAG5)
phenotype	Aox1⁻ His⁺	Aox1⁺ His⁺
state of vector	integrated	autonomous
HBsAg level[a] (shake flask)		
cells/L	10^{11}	10^{11}
monomer (%)	7	1.5
22 nm particle (%)	2.5	0.2
monomer (mg/L)[b]	8.4	1.8
22 nm particle (mg/L)[b]	3	0.24
HBsAg level[a] (fermentor growth)		
cells/l	3.5×10^{12}	8×10^{12}
monomer (%)	7	1
22 nm particle (%)	2.9	0.1
monomer (mg/L)[b]	294	96
22 nm particle (mg/L)[b]	122	10

[a]protein assays; measured by the Bradford Method

[b]HBsAg per liter of culture medium; $OD_{600} = 5 \times 10^7$ cells/mL = 0.14 mg dry weight/mL = 0.06 mg protein/mL

The results presented above demonstrate that high levels of HBsAg can be produced in *Pichia pastoris* when under control of the primary alcohol oxidase gene (AOX1) regulatory region from *Pichia pastoris*.

The examples have been provided merely to illustrate the practice of the invention and should not be read so as to limit the scope of the invention or the appended claims in any way. Reasonable variations and modifications, not departing from the essence and spirit of the invention, are contemplated to be within the scope of patent protection desired and sought.

Claims

Claims for the following Contracting States : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. A DNA fragment comprising:
   (a) an approximately 2.9 kbp long regulatory region derived from the dihydroxyacetone synthase gene (DAS) of Pichia or an approximately 2.0 kbp long regulatory region derived from the primary alcohol oxidase gene (AOX1) of Pichia or an approximately 3 kbp long regulatory region derived from the p40 gene of Pichia, which is capable of controlling the transcription of messenger RNA when positioned at the 5' end of the polypeptide coding region; wherein said regulatory region is responsive to at least one of the conditions selected from:
      (i) the presence of methanol in the culture medium with which a host organism containing said DNA fragment is in contact,
      (ii) the presence of a non-catabolite repressing carbon source other than methanol in the culture medium with which a host organism containing said DNA fragment is in contact, and
      (iii) carbon source starvation in the culture medium with which a host organism containing said DNA fragment is in contact after growth of the host organism on a catabolite repressing carbon and energy source; and
   (b) a polypeptide coding region wherein said coding region codes for hepatitis B surface antigen.

2. The DNA fragment of claim 1 characterized in that said Pichia primary alcohol oxidase (AOX1) regulatory region is identified as shown by the restriction map in Fig. 2 of the drawings.

3. The DNA fragment of claim 1 characterized in that said Pichia DAS regulatory region is identified as shown by the restriction map in Fig. 1 of the drawings.

4. The DNA fragment of claim 1 characterized in that the Pichia p40 regulatory region is identified as shown by the restriction map in Fig. 3 of the drawings.

5. The DNA fragment of claim 1 characterized in that said AOX 1 or p40 or DAS regulatory regions are derived from Pichia pastoris.

6. The DNA fragment of any of claims 1 - 5 further comprising a 3' sequence of DNA downstream of the polypeptide coding region, wherein said 3' sequence of DNA is capable of controlling the polyadenylation and termination of transcription of messenger RNA coded for by said polypeptide coding region.

7. The DNA fragment of any of claims 1 - 6, characterized in that said DNA fragment further comprises one or more additional DNA sequences derived from
      bacterial plasmid DNA,
      bacteriophage DNA,
      yeast plasmid DNA, and
      yeast chromosomal DNA.

8. The DNA fragment of claim 7 wherein said yeast chromosomal DNA comprises an autonomously replicating DNA sequence and a marker gene.

9. The DNA fragment of claim 1 further comprising serially arranged DNA which comprises:
      a first insertable DNA fragment,
      a selectable marker gene, and
      a second insertable DNA fragment;
   wherein said first and second insertable DNA fragments are each at least about 200 nucleotides in length and have nucleotide sequences which are homologous with portions of the genomic DNA of species of the genus Pichia; wherein said DNA fragment and said marker gene are positioned between the 3' end of said first insertable DNA fragment and the 5' end of said second insertable DNA fragment; and wherein said first and second insertable DNA fragments are oriented with respect to one another as they are so oriented in the genome of Pichia.

10. The DNA fragment of claim 1 characterized in that said polypeptide coding region consists essentially of the approximately 700 base pair EcoRI-StuI fragment depicted below:

5'-GAATTCATGG	AGAACATCAC	ATCAGGATTC	CTAGGACCCC
TGCTCGTGTT	ACAGGCGGGG	TTTTTCTTGT	TGACAAGAAT
CCTCACAATA	CCGCAGAGTC	TAGACTCGTG	GTGGACTTCT
CTCAATTTTC	TAGGGGGATC	TCCCGTGTGT	CTTGGCCAAA
ATTCGCAGTC	CCCAACCTCC	AATCACTCAC	CAACCTCCTG
TCCTCCAATT	TGTCCTGGTT	ATCGCTGGAT	GTGTCTGCGG
CGTTTTATCA	TATTCCTCTT	CATCCTGCTG	CTATGCCTCA
TCTTCTTATT	GGTTCTTCTG	GATTATCAAG	GTATGTTGCC
CGTTTGTCCT	CTAATTCCAG	GATCAACAAC	AACCAGTACG
GGACCATGCA	AAACCTGCAC	GACTCCTGCT	CAAGGCAACT
CTATGTTTCC	CTCATGTTGC	TGTACAAAAC	CTACGGATGG
AAATTGCACC	TGTATTCCCA	TCCCATCGTC	CTGGGCTTTC
GCAAAATACC	TATGGGAGTG	GGCCTCAGTC	CGTTTCTCTT
GGCTCAGTTT	ACTAGTGCCA	TTTGTTCAGT	GGTTCGTAGG
GCTTTCCCCC	ACTGTTTGGC	TTTCAGCTAT	ATGGATGATG
TGGTATTGGG	GGCCAAGTCT	GTACAGCATC	GTGAGTCCCT
TTATACCGCT	GTTACCAATT	TTCTTTTGTC	TCTGGGTATA
CATTTAAGGC	CT-3'		

11. A plasmid comprising:
    - the DNA fragment of claim 1,
    - bacterial plasmid DNA,
    - a selectable yeast marker gene, and
    - a yeast autonomous replication sequence.

12. The plasmid of claim 11 characterized in that said plasmid is selected from pBSAG5 (NRRL B-18028), pBSAG5I, as depicted in Fig. 11; and pBSAGI5I (NRRL B-18021), as depicted in Fig. 11, wherein C in Fig. 11 corresponds to a Cla I restriction site and wherein pBSAG5 and pBSAG5I differ in the orientation of the Cla I fragment.

13. An essentially pure culture of a strain of the genus Pichia transformed with a plasmid selected from pBSAG5 (NRRL B-18028), pBSAG5I, and pBSAGI5I (NRRL B-18021) as they are defined in claim 12.

14. The essentially pure culture of claim 13 wherein said strain is of the genus Pichia pastoris.

15. An essentially pure culture of a strain of the genus Pichia transformed with an expression vector comprising the DNA fragment of any of claims 1 - 10.

16. A process for preparing hepatitis B surface antigen characterized by cultivating a Pichia strain transformed with the plasmid of claim 11 in a nutrient medium which comprises at least one carbon and energy source selected from methanol and a catabolite non-repressing carbon source.

17. The process of claim 16 wherein said transformed Pichia strain capable of growth on methanol as a carbon and energy source is Pichia pastoris GS 115.

18. The process of claim 16 wherein said transformed Pichia strain capable of growth on methanol as a carbon and energy source is Pichia pastoris GS 190.

19. The process of claim 16 wherein said transformed Pichia strain is Pichia pastoris PPF 1.

20. The process of claim 16 characterized by
(a) cultivating a yeast strain transformed with the plasmid of claim 11 in a nutrient medium which comprises at least one catabolite repressing carbon and energy source, and
(b) subjecting the product of step (a) to conditions of carbon source starvation.

21. The process of any of claims 16 - 20 further comprising isolating and purifying said hepatitis B surface antigen.

22. A plasmid useful for the construction of expression vectors for the production of heterologous proteins under the control of Pichia primary alcohol oxidase (AOX1) regulatory region obtainable from done pPG4.0 (NRRL B-15868) from the 5'-EcoRI-restriction site to the 3'-SalI-restriction site, wherein starting plasmid pPG2.5 containing said regulatory region is treated as described in Example II of the description to obtain plasmid pAOP2, as depicted in Fig.4.

Claims for the following Contracting States : AT, ES

1. A process for preparing hepatitis B surface antigen characterized by cultivating a Pichia strain transformed with a plasmid containing: a DNA fragment comprising:
(a) an approximately 2.9 kbp long regulatory region derived from the dihydroxyacetone synthase gene (DAS) of Pichia or an approximately 2.0 kbp long regulatory region derived from the primary alcohol oxidase gene (AOX1) of Pichia or an approximately 3 kbp long regulatory region derived from the p40 gene of Pichia, which is capable of controlling the transcription of messenger RNA when positioned at the 5' end of the polypeptide coding region; wherein said regulatory region is responsive to at least one of the conditions selected from:
(i) the presence of methanol in the culture medium with which a host organism containing said DNA fragment is in contact,
(ii) the presence of a non-catabolite repressing carbon source other than methanol in the culture medium with which a host organism containing said DNA fragment is in contact, and
(iii) carbon source starvation in the culture medium with which a host organism containing said DNA fragment is in contact after growth of the host organism on a catabolite repressing carbon and energy source; and
(b) a polypeptide coding region wherein said coding region codes for hepatitis B surface antigen,
bacterial plasmid DNA,
a selectable yeast marker gene, and
a yeast autonomous replication sequence,
in a nutrient medium which comprises at least one carbon and energy source selected from methanol and a catabolite non-repressing carbon source.

2. The process of claim 1 characterized in that said Pichia primary alcohol oxidase (AOX1) regulatory region is identified as shown by the restriction map in Fig. 2 of the drawings.

3. The process of claim 1 characterized in that said Pichia DAS regulatory region is identified as shown by the restriction map in Fig. 1 of the drawings.

4. The process of claim 1 characterized in that the Pichia p40 regulatory region is identified as shown by the restriction map in Fig. 3 of the drawings.

5. The process of claim 1 characterized in that said AOX 1 or p40 or DAS regulatory regions are derived from Pichia pastoris.

6. The process of any of claims 1 - 5 wherein said DNA fragment further comprises a 3' sequence of DNA downstream of the polypeptide coding region, wherein said 3' sequence of DNA is capable of controlling the polyadenylation and termination of transcription of messenger RNA coded for by said

polypeptide coding region.

7. The process of any of claims 1 - 6, characterized in that said DNA fragment further comprises one or more additional DNA sequences derived from
   bacterial plasmid DNA,
   bacteriophage DNA,
   yeast plasmid DNA, and
   yeast chromosomal DNA.

8. The process of claim 7 wherein said yeast chromosomal DNA comprises an autonomously replicating DNA sequence and a marker gene.

9. The process of claim 1 wherein said DNA fragment further comprises serially arranged DNA which comprises:
   a first insertable DNA fragment,
   a selectable marker gene, and
   a second insertable DNA fragment;
   wherein said first and second insertable DNA fragments are each at least about 200 nucleotides in length and have nucleotide sequences which are homologous with portions of the genomic DNA of species of the genus Pichia; wherein said DNA fragment and said marker gene are positioned between the 3' end of said first insertable DNA fragment and the 5' end of said second insertable DNA fragment; and wherein said first and second insertable DNA fragments are oriented with respect to one another as they are so oriented in the genome of Pichia.

10. The process of claim 1 characterized in that said polypeptide coding region consists essentially of the approximately 700 base pair EcoRI-StuI fragment depicted below:

```
5'-GAATTCATGG   AGAACATCAC   ATCAGGATTC   CTAGGACCCC
   TGCTCGTGTT   ACAGGCGGGG   TTTTTCTTGT   TGACAAGAAT
   CCTCACAATA   CCGCAGAGTC   TAGACTCGTG   GTGGACTTCT
   CTCAATTTTC   TAGGGGGATC   TCCCGTGTGT   CTTGGCCAAA
   ATTCGCAGTC   CCCAACCTCC   AATCACTCAC   CAACCTCCTG
   TCCTCCAATT   TGTCCTGGTT   ATCGCTGGAT   GTGTCTGCGG
   CGTTTTATCA   TATTCCTCTT   CATCCTGCTG   CTATGCCTCA
   TCTTCTTATT   GGTTCTTCTG   GATTATCAAG   GTATGTTGCC
   CGTTTGTCCT   CTAATTCCAG   GATCAACAAC   AACCAGTACG
   GGACCATGCA   AAACCTGCAC   GACTCCTGCT   CAAGGCAACT
   CTATGTTTCC   CTCATGTTGC   TGTACAAAAC   CTACGGATGG
   AAATTGCACC   TGTATTCCCA   TCCCATCGTC   CTGGGCTTTC
   GCAAAATACC   TATGGGAGTG   GGCCTCAGTC   CGTTTCTCTT
   GGCTCAGTTT   ACTAGTGCCA   TTTGTTCAGT   GGTTCGTAGG
   GCTTTCCCCC   ACTGTTTGGC   TTTCAGCTAT   ATGGATGATG
   TGGTATTGGG   GGCCAAGTCT   GTACAGCATC   GTGAGTCCCT
   TTATACCGCT   GTTACCAATT   TTCTTTTGTC   TCTGGGTATA
   CATTTAAGGC   CT-3'
```

11. The process of claim 1 characterized in that said plasmid is selected from
pBSAG5 (NRRL B-18028),
pBSAG5I, as depicted in Fig. 11; and
pBSAGI5I (NRRL B-18021), as depicted in Fig. 11, wherein C in Fig. 11 corresponds to a Cla I restriction site and wherein pBSAG5 and pBSAG5I differ in the orientation of the Cla I fragment.

12. An essentially pure culture of a strain of the genus Pichia transformed with a plasmid selected from pBSAG5 (NRRL B-18028), pBSAG5I, and pBSAGI5I (NRRL B-18021) as they are defined in claim 11.

13. The essentially pure culture of claim 12 wherein said strain is of the genus Pichia pastoris.

14. An essentially pure culture of a strain of the genus Pichia transformed with an expression vector comprising the DNA fragment as defined in any of claims 1 - 10.

15. The process of claim 1 wherein said transformed Pichia strain capable of growth on methanol as a carbon and energy source is Pichia pastoris GS 115.

16. The process of claim 1 wherein said transformed Pichia strain capable of growth on methanol as a carbon and energy source is Pichia pastoris GS 190.

17. The process of claim 1 wherein said transformed Pichia strain is Pichia pastoris PPF 1.

18. The process of claim 1 characterized by
(a) cultivating a yeast strain transformed with the plasmid of claim 1 in a nutrient medium which comprises at least one catabolite repressing carbon and energy source, and
(b) subjecting the product of step (2) to conditions of carbon source starvation.

19. The process of any claims 1 - 18 further comprising isolating and purifying said hepatitis B surface antigen.

20. A process for preparing a plasmid useful for the construction of expression vectors for the production of heterologons proteins under the control of Pichia primary alcohol oxidase (AOX1) regulatory region obtainable from clone pPG4.0 (NRRL B-15868) from the 5'-EcoRI-restriction site to the 3'-SalI-restriction site, wherein starting plasmid pPG2.5 containing said regulatory region is treated as described in Example II of the description to obtain plasmid pAOP2, as depicted in Fig.4.

Patentansprüche
Patentansprüche für folgende Vertragsstaaten : BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. Ein DNA-Fragment, umfassend:
(a) eine ungefähr 2,9 kbp lange, aus dem Dihydroxyacetonsynthase (DAS)-Gen von Pichia abgeleitete regulatorische Region oder eine ungefähr 2,0 kbp lange, aus dem primären Alkoholoxidase-Gen (AOX1) von Pichia abgeleitete regulatorische Region oder eine ungefähr 3 kbp lange, aus dem p40-Gen von Pichia abgeleitete regulatorische Region, die fähig ist, die Transkription von Messenger-RNA zu kontrollieren, wenn sie am 5'-Ende der für ein Polypeptid codierenden Region angeordnet ist, wobei die regulatorische Region auf mindestens eine der folgenden Bedingungen anspricht:
(i) die Gegenwart von Methanol im Kulturmedium, mit dem ein das DNA-Fragment enthaltender Wirtsorganismus in Kontakt steht,
(ii) die Gegenwart einer nicht katabolisch reprimierenden Kohlenstoffquelle außer Methanol im Kulturmedium, mit dem ein das DNA-Fragment enthaltender Wirtsorganismus in Kontakt steht, und
(iii) ein Mangel an Kohlenstoffquellen im Kulturmedium, mit dem ein das DNA-Fragment enthaltender Wirtsorganismus in Kontakt steht, nachdem der Wirtsorganismus auf einer katabolisch reprimierenden Kohlenstoff- und Energiequelle gewachsen ist; und
(b) eine für ein Polypeptid codierende Region, wobei die codierende Region für Hepatitis B-Oberflächen-Antigen codiert.

2. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß die regulatorische Region für primäre Alkoholoxidase (AOX1) von Pichia durch die in Fig. 2 der Zeichnungen gezeigte Restriktionskarte identifiziert ist.

3. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß die regulatorische Region für DAS aus Pichia durch die in Fig. 1 der Zeichnungen gezeigte Restriktionskarte identifiziert ist.

4. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß die regulatorische, Region für p40 aus Pichia durch die in Fig. 3 der Zeichnungen gezeigte Restriktionskarte identifiziert ist.

5. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß die regulatorische Region für AOX1 oder p40 oder DAS aus Pichia pastoris abgeleitet ist.

6. DNA-Fragment nach einem der Ansprüche 1-5, ferner umfassend eine 3'-Sequenz von DNA in downstream-Richtung von der für das Polypeptid codierenden Region, wobei die 3'-Sequenz von DNA zur Kontrolle der Polyadenylierung und Termination der Transkription von Messenger-RNA, für die die für das Polypeptid codierende Region codiert, fähig ist.

7. DNA-Fragment nach einem der Ansprüche 1-6, dadurch gekennzeichnet, daß das DNA-Fragment ferner eine oder mehrere zusätzliche DNA Sequenzen umfaßt, die aus
bakterieller Plasmid-DNA,
Bakteriophagen-DNA,
Hefeplasmid-DNA und
chromosomaler Hefe-DNA abgeleitet sind.

8. DNA-Fragment nach Anspruch 7, wobei die chromosomale Hefe-DNA eine autonom replizierende DNA-Sequenz und ein Markergen umfaßt.

9. DNA-Fragment nach Anspruch 1, ferner umfassend nacheinander angeordnete DNA, die
ein erstes inserierbares DNA-Fragment,
ein selektierbares Markergen und
ein zweites inserierbares DNA-Fragment umfaßt,
wobei das erste und das zweite inserierbare DNA-Fragment jeweils eine Länge von mindestens 200 Nucleotiden aufweisen und wobei sie Nucleotidsequenzen besitzen, die homolog zu Abschnitten der genomischen DNA von Spezies der Gattung Pichia sind, wobei das DNA-Fragment und das Markergen zwischen dem 3'-Ende des ersten inserierbaren DNA-Fragments und dem 5'-Ende des zweiten inserierbaren DNA-Fragments angeordnet sind und wobei das erste und das zweite inserierbare DNA-Fragment so zueinander angeordnet sind, wie sie im Genom von Pichia angeordnet sind.

10. DNA-Fragment nach Anspruch 1, dadurch gekennzeichnet, daß die für das Polypeptid codierende Region im wesentlichen aus dem ungefähr 700 Basenpaare langen EcoRI-StuI-Fragment besteht, das nachstehend gezeigt ist:

5'-GAATTCATGG	AGAACATCAC	ATCAGGATTC	CTAGGACCCC
TGCTCGTGTT	ACAGGCGGGG	TTTTTCTTGT	TGACAAGAAT
CCTCACAATA	CCGCAGAGTC	TAGACTCGTG	GTGGACTTCT
CTCAATTTTC	TAGGGGGATC	TCCCGTGTGT	CTTGGCCAAA
ATTCGCAGTC	CCCAACCTCC	AATCACTCAC	CAACCTCCTG
TCCTCCAATT	TGTCCTGGTT	ATCGCTGGAT	GTGTCTGCGG
CGTTTTATCA	TATTCCTCTT	CATCCTGCTG	CTATGCCTCA
TCTTCTTATT	GGTTCTTCTG	GATTATCAAG	GTATGTTGCC
CGTTTGTCCT	CTAATTCCAG	GATCAACAAC	AACCAGTACG
GGACCATGCA	AAACCTGCAC	GACTCCTGCT	CAAGGCAACT
CTATGTTTCC	CTCATGTTGC	TGTACAAAAC	CTACGGATGG
AAATTGCACC	TGTATTCCCA	TCCCATCGTC	CTGGGCTTTC
GCAAAATACC	TATGGGAGTG	GGCCTCAGTC	CGTTTCTCTT
GGCTCAGTTT	ACTAGTGCCA	TTTGTTCAGT	GGTTCGTAGG
GCTTTCCCCC	ACTGTTTGGC	TTTCAGCTAT	ATGGATGATG
TGGTATTGGG	GGCCAAGTCT	GTACAGCATC	GTGAGTCCCT
TTATACCGCT	GTTACCAATT	TTCTTTTGTC	TCTGGGTATA
CATTTAAGGC	CT-3'		

11. Plasmid, umfassend:
    das DNA-Fragment nach Anspruch 1,
    bakterielle Plasmid-DNA,
    ein selektierbares Hefe-Markergen und
    eine autonom replizierende Hefe-Sequenz.

12. Plasmid nach Anspruch 11, dadurch gekennzeichnet, daß das Plasmid ausgewählt ist aus
    pBSAG5 (NRRL B-18028),
    pBSAG5I, wie in Fig. 11 gezeigt, und
    pBSAGI5I (NRRL B-18021), wie in Fig. 11 gezeigt, wobei C in Fig. 11 der Restriktionsstelle ClaI entspricht und wobei pBSAG5 und pBSAG5I sich in der Orientierung des ClaI-Fragments unterscheiden.

13. Eine im wesentlichen reine Kultur eines Stammes der Gattung Pichia, transformiert mit einem Plasmid, ausgewählt aus pBSAG5 (NRRL B-18028), pBSAG5I und pBSAGI5I (NRRL B-18021), wie sie in Anspruch 12 definiert sind.

14. Im wesentlichen reine Kultur nach Anspruch 13, wobei der Stamm zur Gattung Pichia pastoris gehört.

15. Eine im wesentlichen reine Kultur eines Stammes der Gattung Pichia, transformiert mit einem Expressionsvektor, der das DNA-Fragment nach einem der Ansprüche 1 bis 10 umfaßt.

16. Verfahren zur Herstellung eines Hepatitis B-Oberflächen-Antigens, gekennzeichnet durch Züchten eines Pichia-Stamms, der mit einem Plasmid nach Anspruch 11 transformiert worden ist, in einem Nährmedium, das mindestens eine Kohlenstoff- und Energiequelle, ausgewählt aus Methanol und katabolisch nicht reprimierenden Kohlenstoffquellen, umfaßt.

17. Verfahren nach Anspruch 16, wobei es sich bei dem transformierten Pichia-Stamm, der zum Wachstum auf Methanol als Kohlenstoff- und Energiequelle fähig ist, um Pichia pastoris GS115 handelt.

18. Verfahren nach Anspruch 16, wobei es sich bei dem Pichia-Stamm, der zum Wachstum auf Methanol als Kohlenstoff- und Energiequelle fähig ist, um Pichia pastoris GS190 handelt.

19. Verfahren nach Anspruch 16, wobei es sich bei dem transformierten Pichia-Stamm um Pichia pastoris PPF1 handelt.

20. Verfahren nach Anspruch 16, gekennzeichnet durch
(a) Züchten eines Hefestamms, der mit dem Plasmid nach Anspruch 11 transformiert wurde, in einem Nährmedium, das mindestens eine katabolisch reprimierende Kohlenstoff- und Energiequelle umfaßt, und
(b) Unterwerfen des Produkts aus Stufe (a) unter Bedingungen eines Mangels an Kohlenstoffquellen.

21. Verfahren nach einem der Ansprüche 16 bis 20, ferner umfassend das Isolieren und Reinigen des Hepatitis B-Oberflächen-Antigens.

22. Ein Plasmid, geeignet für die Konstruktion von Expressionsvektoren für die Herstellung heterologer Proteine unter der Kontrolle der regulatorischen Region von primärer Alkoholoxidase (AOX1) aus Pichia, erhältlich aus dem Klon pPG4.0 (NRRL B-15868) von der 5'-EcoRI-Restriktionsstelle bis zur 3'-SalI-Restriktionsstelle, wobei das Ausgangsplasmid pPG2.5, das die regulatorische Region enthält, gemäß den Angaben in Beispiel II behandelt wird, um das in Fig. 4 dargestellte Plasmid pAOP2 zu erhalten.

Patentansprüche für folgende Vertragsstaaten : AT, ES

1. Verfahren zur Herstellung eines Hepatitis B-Oberflächen-Antigens, gekennzeichnet durch Züchten eines Pichia-Stamms, der mit einem Plasmid transformiert worden ist, das folgendes enthält:
ein DNA-Fragment, umfassend:
(a) eine ungefähr 2,9 kbp lange, aus dem Dihydroxyacetonsynthase (DAS)-Gen von Pichia abgeleitete regulatorische Region oder eine ungefähr 2,0 kbp lange, aus dem primären Alkoholoxidase-Gen (AOX1) von Pichia abgeleitete regulatorische Region oder eine ungefähr 3 kbp lange, aus dem p40-Gen von Pichia abgeleitete regulatorische Region, die fähig ist, die Transkription von Messenger-RNA zu kontrollieren, wenn sie am 5'-Ende der für ein Polypeptid codierenden Region angeordnet ist, wobei die regulatorische Region auf mindestens eine der folgenden Bedingungen anspricht:
(i) die Gegenwart von Methanol im Kulturmedium, mit dem ein das DNA-Fragment enthaltender Wirtsorganismus in Kontakt steht,
(ii) die Gegenwart einer nicht katabolisch reprimierenden Kohlenstoffquelle außer Methanol im Kulturmedium, mit dem ein das DNA-Fragment enthaltender Wirtsorganismus in Kontakt steht, und
(iii) ein Mangel an Kohlenstoffquellen im Kulturmedium, mit dem ein das DNA-Fragment enthaltender Wirtsorganismus in Kontakt steht, nachdem der Wirtsorganismus auf einer katabolisch reprimierenden Kohlenstoff- und Energiequelle gewachsen ist; und
(b) eine für ein Polypeptid codierende Region, wobei die codierende Region für Hepatitis B-Oberflächen-Antigen codiert,
bakterielle Plasmid-DNA,
ein selektierbares Hefe-Markergen und
eine autonom replizierende Hefe-Sequenz,
in einem Nährmedium, das mindestens eine Kohlenstoff- und Energiequelle, ausgewählt aus Methanol und katabolisch nicht reprimierenden Kohlenstoffquellen, umfaßt.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die regulatorische Region für primäre Alkoholoxidase (AOX1) von Pichia durch die in Fig. 2 der Zeichnungen gezeigte Restriktionskarte identifiziert ist.

3. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die regulatorische Region für DAS aus Pichia durch die in Fig. 1 der Zeichnungen gezeigte Restriktionskarte identifiziert ist.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die regulatorische Region für p40 aus Pichia durch die in Fig. 3 der Zeichnungen gezeigte Restriktionskarte identifiziert ist.

5. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die regulatorische Region für AOX1 oder p40 oder DAS aus Pichia pastoris abgeleitet ist.

6. Verfahren nach einem der Ansprüche 1-5, wobei das DNA-Fragment ferner eine 3'-Sequenz von DNA in downstream-Richtung von der für das Polypeptid codierenden Region umfaßt, wobei die 3'-Sequenz von DNA zur Kontrolle der Polyadenylierung und Termination der Transkription von Messenger-RNA, für die die für das Polypeptid codierende Region codiert, fähig ist.

7. Verfahren nach einem der Ansprüche 1-6, dadurch gekennzeichnet, daß das DNA-Fragment ferner eine oder mehrere zusätzliche DNA Sequenzen umfaßt, die aus
   bakterieller Plasmid-DNA,
   Bakteriophagen-DNA,
   Hefeplasmid-DNA und
   chromosomaler Hefe-DNA abgeleitet sind.

8. Verfahren nach Anspruch 7, wobei die chromosomale Hefe-DNA eine autonom replizierende DNA-Sequenz und ein Markergen umfaßt.

9. Verfahren nach Anspruch 1, wobei das DNA-Fragment ferner nacheinander angeordnete DNA umfaßt, die
   ein erstes inserierbares DNA-Fragment,
   ein selektierbares Markergen und
   ein zweites inserierbares DNA-Fragment umfaßt,
   wobei das erste und das zweite inserierbare DNA-Fragment jeweils eine Länge von mindestens 200 Nucleotiden aufweisen und wobei sie Nucleotidsequenzen besitzen, die homolog zu Abschnitten der genomischen DNA von Spezies der Gattung Pichia sind, wobei das DNA-Fragment und das Markergen zwischen dem 3'-Ende des ersten inserierbaren DNA-Fragments und dem 5'-Ende des zweiten inserierbaren DNA-Fragments angeordnet sind und wobei das erste und das zweite inserierbare DNA-Fragment so zueinander angeordnet sind, wie sie im Genom von Pichia angeordnet sind.

10. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß die für das Polypeptid codierende Region im wesentlichen aus dem ungefähr 700 Basenpaare langen EcoRI-StuI-Fragment besteht, das nachstehend gezeigt ist:

5'-GAATTCATGG	AGAACATCAC	ATCAGGATTC	CTAGGACCCC
TGCTCGTGTT	ACAGGCGGGG	TTTTTCTTGT	TGACAAGAAT
CCTCACAATA	CCGCAGAGTC	TAGACTCGTG	GTGGACTTCT
CTCAATTTTC	TAGGGGGATC	TCCCGTGTGT	CTTGGCCAAA
ATTCGCAGTC	CCCAACCTCC	AATCACTCAC	CAACCTCCTG
TCCTCCAATT	TGTCCTGGTT	ATCGCTGGAT	GTGTCTGCGG
CGTTTTATCA	TATTCCTCTT	CATCCTGCTG	CTATGCCTCA
TCTTCTTATT	GGTTCTTCTG	GATTATCAAG	GTATGTTGCC
CGTTTGTCCT	CTAATTCCAG	GATCAACAAC	AACCAGTACG
GGACCATGCA	AAACCTGCAC	GACTCCTGCT	CAAGGCAACT
CTATGTTTCC	CTCATGTTGC	TGTACAAAAC	CTACGGATGG
AAATTGCACC	TGTATTCCCA	TCCCATCGTC	CTGGGCTTTC
GCAAAATACC	TATGGGAGTG	GGCCTCAGTC	CGTTTCTCTT
GGCTCAGTTT	ACTAGTGCCA	TTTGTTCAGT	GGTTCGTAGG
GCTTTCCCCC	ACTGTTTGGC	TTTCAGCTAT	ATGGATGATG
TGGTATTGGG	GGCCAAGTCT	GTACAGCATC	GTGAGTCCCT
TTATACCGCT	GTTACCAATT	TTCTTTTGTC	TCTGGGTATA
CATTTAAGGC	CT-3'		

11. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Plasmid ausgewählt ist aus
pBSAG5 (NRRL B-18028),
pBSAG5I, wie in Fig. 11 gezeigt, und
pBSAGI5I (NRRL B-18021), wie in Fig. 11 gezeigt, wobei C in Fig. 11 der Restriktionsstelle ClaI entspricht und wobei pBSAG5 und pBSAG5I sich in der Orientierung des ClaI-Fragments unterscheiden.

12. Eine im wesentlichen reine Kultur eines Stammes der Gattung Pichia, transformiert mit einem Plasmid, ausgewählt aus pBSAG5 (NRRL B-18028), pBSAG5I und pBSAGI5I (NRRL B-18021), wie sie in Anspruch 11 definiert sind.

13. Im wesentlichen reine Kultur nach Anspruch 12, wobei der Stamm zur Gattung Pichia pastoris gehört.

14. Eine im wesentlichen reine Kultur eines Stammes der Gattung Pichia, transformiert mit einem Expressionsvektor, der das DNA-Fragment umfaßt, wie es in einem der Ansprüche 1 bis 10 definiert ist.

15. Verfahren nach Anspruch 1, wobei es sich bei dem transformierten Pichia-Stamm, der zum Wachstum auf Methanol als Kohlenstoff- und Energiequelle fähig ist, um Pichia pastoris GS115 handelt.

16. Verfahren nach Anspruch 1, wobei es sich bei dem Pichia-Stamm, der zum Wachstum auf Methanol als Kohlenstoff- und Energiequelle fähig ist, um Pichia pastoris GS190 handelt.

17. Verfahren nach Anspruch 1, wobei es sich bei dem transformierten Pichia-Stamm um Pichia pastoris PPF1 handelt.

18. Verfahren nach Anspruch 1, gekennzeichnet durch
(a) Züchten eines Hefestamms, der mit dem Plasmid nach Anspruch 1 transformiert worden ist, in einem Nährmedium, das mindestens eine katabolisch reprimierende Kohlenstoff- und Energiequelle

umfaßt, und
(b) Unterwerfen des Produkts aus Stufe (a) unter Bedingungen eines Mangels an Kohlenstoffquellen.

19. Verfahren nach einem der Ansprüche 1 bis 18, ferner umfassend das Isolieren und Reinigen des Hepatitis B-Oberflächen-Antigens.

20. Verfahren zur Herstellung eines Plasmids, geeignet für die Konstruktion von Expressionsvektoren für die Herstellung heterologer Proteine unter der Kontrolle der regulatorischen Region von primärer Alkoholoxidase (AOX1) aus Pichia, erhältlich aus dem Klon pPG4.0 (NRRL B-15868) von der 5'-EcoRI-Restriktionsstelle bis zur 3'-SalI-Restriktionsstelle, wobei das Ausgangsplasmid pPG2.5, das die regulatorische Region enthält, gemäß den Angaben in Beispiel II behandelt wird, um das in Fig. 4 gezeigte Plasmid pAOP2 zu erhalten.

Revendications
Revendications pour les Etats contractants suivants : BE, CH, DE, FR, GB, GR, IT, IT, LI, LU, NL, SE

1. Fragment d'ADN comportant :
(a) une région régulatrice approximativement de 2,9 kbp de long dérivée à partir du gène (DAS) de dihydroxyacétone synthase du *Pichia* ou une région régulatrice approximativement de 2,0 kbp de long dérivée du gène (AOX1) d'alcool primaire oxydase du *Pichia* ou une région régulatrice d'approximativement 3 kbp de long dérivée du gène p40 du *Pichia*, qui est capable de commander la transcription de l'ARN messager lorsqu'il est situé à l'extrémité 5' de la région de codage du polypeptide, dans lequel ladite région régulatrice est sensible à au moins une des conditions choisies parmi :
(i) la présence de méthanol dans le milieu de culture avec lequel un organisme hôte contenant ledit fragment d'ADN est en contact,
(ii) la présence d'une source de carbone ne réprimant pas les catabolites autre que le méthanol dans le milieu de culture avec lequel un organisme hôte contenant ledit fragment d'ADN est en contact, et
(iii) la privation de source de carbone dans le milieu de culture avec lequel un organisme hôte contenant ledit fragment d'ADN est en contact après croissance de l'organisme hôte sur un carbone réprimant les catabolites, et de source d'énergie, et
(b) une région de codage de polypeptide dans laquelle ladite région de codage code l'antigène de surface de l'hépatite B.

2. Fragment d'ADN selon la revendication 1, caractérisé en ce que ladite région régulatrice du gène (AOX1) d'alcool primaire oxydase de *Pichia* est identifiée comme représenté par la carte de restriction de la figure 2 des dessins.

3. Fragment d'ADN selon la revendication 1, caractérisé en ce que ladite région régulatrice DAS du *Pichia* est identifiée comme représenté par la carte de restriction de la figure 1 des dessins.

4. Fragment d'ADN selon la revendication 1, caractérisé en ce que la région régulatrice p40 du *Pichia* est identifiée comme représenté par la carte de restriction de la figure 3 des dessins.

5. Fragment d'ADN selon la revendication 1, caractérisé en ce que lesdites régions régulatrices DAS ou AOX1 ou p40 sont dérivées du *Pichia pastoris*.

6. Fragment d'ADN selon l'une quelconque des revendications 1 à 5, comportant en outre une séquence 3' d'ADN en aval de la région de codage de polypeptide, dans lequel ladite séquence 3' d'ADN est capable de commander la polyadénylation et l'extrémité de transcription de l'ARN messager codé pour ladite région de codage de polypeptide.

7. Fragment d'ADN selon l'une quelconque des revendications 1 à 6, caractérisé en ce que ledit fragment d'ADN comporte en outre une ou plusieurs séquences ADN supplémentaires dérivées de
l'ADN plasmide bactérien,
l'ADN bactériophage,
l'ADN plasmide de levure, et

l'ADN chromosomal de levure

8. Fragment d'ADN selon la revendication 7, dans lequel ledit ADN chromosomal de levure comporte une séquence ADN de réplication autonome et un gène formant marqueur.

9. Fragment d'ADN selon la revendication 1, comportant en outre de l'ADN en série qui comporte :
un premier fragment d'ADN insérable,
un gène marqueur pouvant être choisi, et
un second fragment d'ADN insérable;
dans lequel lesdits premier et second fragments d'ADN insérables sont chacun au moins d'environ 200 nucléotides en longueur et ont des séquences nucléotidiques homologues à des parties de l'ADN génomique des espèces du genre *Pichia*; dans lequel ledit fragment d'ADN et ledit gène formant marqueur sont positionnés entre l'extrémité 3' dudit premier fragment d'ADN insérable et l'extrémité 5' dudit second fragment d'ADN insérable, et dans lequel lesdits premier et second fragments sont orientés l'un par rapport à l'autre comme ils sont orientés dans le génome du *Pichia*.

10. Fragment d'ADN selon la revendication 1, caractérisé en ce que ladite région de codage de polypeptide est constituée essentiellement d'un fragment *Eco*RI-*Stf*I d'approximativement 700 paires de base décrit ci-dessous :

```
5'-GAATTCATGG  AGAACATCAC  ATCAGGATTC  CTAGGACCCC
   TGCTCGTGTT  ACAGGCGGGG  TTTTTCTTGT  TGACAAGAAT
   CCTCACAATA  CCGCAGAGTC  TAGACTCGTG  GTGGACTTCT
   CTCAATTTTC  TAGGGGGATC  TCCCGTGTGT  CTTGGCCAAA
   ATTCGCAGTC  CCCAACCTCC  AATCACTCAC  CAACCTCCTG
   TCCTCCAATT  TGTCCTGGTT  ATCGCTGGAT  GTGTCTGCGG
   CGTTTTATCA  TATTCCTCTT  CATCCTGCTG  CTATGCCTCA
   TCTTCTTATT  GGTTCTTCTG  GATTATCAAG  GTATGTTGCC
   CGTTTGTCCT  CTAATTCCAG  GATCAACAAC  AACCAGTACG
   GCACCATGCA  AAACCTGCAC  GACTCCTGCT  CAAGGCAACT
   CTATGTTTCC  CTCATGTTGC  TGTACAAAAC  CTACGGATGG
   AAATTGCACC  TGTATTCCCA  TCCCATCGTC  CTGGGCTTTC
   GCAAAATACC  TATGGGAGTG  GGCCTCAGTC  CGTTTCTCTT
   GGCTCAGTTT  ACTAGTGCCA  TTTGTTCAGT  GGTTCGTAGG
   GCTTTCCCCC  ACTGTTTGGC  TTTCAGCTAT  ATGGATGATG
   TGGTATTGGG  GGCCAAGTCT  GTACAGCATC  GTGAGTCCCT
   TTATACCGCT  GTTACCAATT  TTCTTTTGTC  TCTGGGTATA
   CATTTAAGGC  CT-3'
```

11. Plasmide comportant :
l'ADN de plasmide bactérien,
un gène formant marqueur de levure pouvant être choisi, et
une séquence de réplication autonome de la levure.

12. Plasmide selon la revendication 11, caractérisé en ce que ledit plasmide est choisi parmi
pBSAG5 (NRRL B-18028),
pBSAG5I, comme représenté sur la figure 11; et
pBSAGI5I (NRRL B-18021), comme représenté sur la figure 11, dans lequel C sur la figure 11

correspond à un site de restriction *Cla* I et dans lequel pBSAG5 et pBSAG5I ont une orientation différente du fragment *Cla* I.

13. Culture à peu près pure d'une souche du genre *Pichia* transformé à l'aide d'un plasmide choisi parmi pBSAG5 (NRRL B-18028), pBSAG5I, et pBSAGI5I (NRRL B-18021) tels que définis dans la revendication 12.

14. Culture à peu près pure selon la revendication 13, dans laquelle ladite souche est du genre *Pichia pastoris*.

15. Culture à peu près pure d'une souche du genre *Pichia* transformé à l'aide d'un vecteur d'expression comportant le fragment d'ADN de l'une quelconque des revendications 1 à 10.

16. Procédé de préparation de l'antigène de surface de l'hépatite B, caractérisé en ce qu'on cultive une souche de *Pichia* transformé à l'aide du plasmide de la revendication 11 dans un milieu nutritif qui est constitué au moins d'un carbone et d'une source d'énergie choisis parmi le méthanol et une source de carbone ne réprimant pas les catabolites.

17. Procédé selon la revendication 16, dans lequel ladite souche de *Pichia* transformé capable de croître sur du méthanol en tant que source de carbone et d'énergie est le *Pichia pastoris* GS 115.

18. Procédé selon la revendication 16, dans lequel ladite souche de *Pichia* transformé capable de croître sur du méthanol en tant que source de carbone et d'énergie est le *Pichia pastoris* GS 190.

19. Procédé selon la revendication 16, dans lequel ladite souche de *Pichia transformé* est du *Pichia pastoris* PPF 1.

20. Procédé selon la revendication 16, caractérisé en ce que :
   (a) on cultive une souche de levure transformée à l'aide du plasmide de la revendication 11 dans un milieu nutritif qui est constitué au moins d'une source de carbone réprimant les catabolites et d'une source d'énergie, et
   (b) on soumet le produit de l'étape (a) à des conditions de privation de source de carbone.

21. Procédé selon l'une quelconque des revendications 16 à 20 comportant en outre l'isolation et la purification dudit antigène de surface de l'hépatite B.

22. Plasmide utile pour la construction des vecteurs d'expression pour la production de protéines hétérologues sous le contrôle d'une région régulatrice de l'alcool primaire oxydase du *Pichia* (AOX1) pouvant être obtenue à partir du clone pPG4.0 (NRRL B-15868) à partir du site de restriction 5'-*Eco*RI vers le site de restriction 3'-*Sal*I dans lequel le démarrage du plasmide pPG25 contenant ladite région régulatrice est traité comme décrit dans l'exemple II de la description pour obtenir le plasmide pAOP2, comme décrit sur la figure 4.

Revendications pour les Etats contractants suivants : AT, ES

1. Procédé de préparation d'antigène de surface de l'hépatite B, caractérisé en ce qu'on cultive une souche de *Pichia* transformé à l'aide d'un plasmide contenant un fragment d'ADN comportant :
   (a) une région régulatrice approximativement de 2,9 kbp de long dérivée à partir du gène (DAS) de dihydroxyacétone synthase du *Pichia* ou une région régulatrice approximativement de 2,0 kbp de long dérivée du gène (AOX1) d'alcool primaire oxydase du *Pichia* ou une région régulatrice d'approximativement 3 kbp de long dérivée du gène p40 du *Pichia*, qui est capable de commander la transcription de l'ARN messager lorsqu'il est situé à l'extrémité 5' de la région de codage du polypeptide, dans lequel ladite région régulatrice est sensible à au moins une des conditions choisies parmi :
      (i) la présence de méthanol dans le milieu de culture avec lequel un organisme hôte contenant ledit fragment d'ADN est en contact,
      (ii) la présence d'une source de carbone ne réprimant pas les catabolites autre que le méthanol dans le milieu de culture avec lequel un organisme hôte contenant ledit fragment d'ADN est en

contact, et

(iii) la privation de source de carbone dans le milieu de culture avec lequel un organisme hôte contenant ledit fragment d'ADN est en contact après croissance de l'organisme hôte sur un carbone réprimant les catabolites, et de source d'énergie, et

(b) une région de codage de polypeptide dans laquelle ladite région de codage code l'antigène de surface de l'hépatite B, de l'ADN de plasmide bactérien, un gène formant marqueur de levure pouvant être choisi, et une séquence de réplication autonome de la levure, dans un milieu nutritif qui est constitué au moins d'un carbone et d'une source d'énergie choisis parmi le méthanol et une source de carbone ne réprimant pas les catabolites.

2. Procédé selon la revendication 1, caractérisé en ce que ladite région régulatrice du gène (AOX1) d'alcool primaire oxydase de *Pichia* est identifiée comme représenté par la carte de restriction de la figure 2 des dessins.

3. Procédé selon la revendication 1, caractérisé en ce que ladite région régulatrice DAS du *Pichia* est identifiée comme représenté par la carte de restriction de la figure 1 des dessins.

4. Procédé selon la revendication 1, caractérisé en ce que la région régulatrice p40 du *Pichia* est identifiée comme représenté par la carte de restriction de la figure 3 des dessins.

5. Procédé selon la revendication 1, caractérisé en ce que lesdites régions régulatrices DAS ou AOX1 ou p40 sont dérivées du *Pichia pastoris*.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel ledit fragment d'ADN comporte en outre une séquence 3' d'ADN en aval de la région de codage de polypeptide, dans lequel ladite séquence 3' d'ADN est capable de commander la polyadénylation et l'extrémité de transcription de l'ARN messager codé pour ladite région de codage de polypeptide.

7. Procédé selon l'une quelconque des revendications 1 à 6, caractérisé en ce que ledit fragment d'ADN comporte en outre une ou plusieurs séquences ADN supplémentaires dérivées de
l'ADN plasmide bactérien,
l'ADN bactériophage,
l'ADN plasmide de levure, et
l'ADN chromosomal de levure

8. Procédé selon la revendication 7, dans lequel ledit ADN chromosomal de levure comporte une séquence ADN de réplication autonome et un gène formant marqueur.

9. Procédé selon la revendication 1, dans lequel ledit fragment d'ADN comporte en outre de l'ADN en série qui comporte :
un premier fragment d'ADN insérable,
un gène marqueur pouvant être choisi, et
un second fragment d'ADN insérable;
dans lequel lesdits premier et second fragments d'ADN insérables sont chacun au moins d'environ 200 nucléotides en longueur et ont des séquences nucléotidiques homologues à des parties de l'ADN génomique des espèces du genre *Pichia*; dans lequel ledit fragment d'ADN et ledit gène formant marqueur sont positionnés entre l'extrémité 3' dudit premier fragment d'ADN insérable et l'extrémité 5' dudit second fragment d'ADN insérable, et dans lequel lesdits premier et second fragments sont orientés l'un par rapport à l'autre comme ils sont orientés dans le génome du *Pichia*.

10. Procédé selon la revendication 1, caractérisé en ce que ladite région de codage de polypeptide est constituée essentiellement d'un fragment *Eco*RI-*Stf*I d'approximativement 700 paires de base décrit ci-dessous:

```
5'-GAATTCATGG  AGAACATCAC  ATCAGGATTC  CTAGGACCCC
   TGCTCGTGTT  ACAGGCGGGG  TTTTTCTTGT  TGACAAGAAT
   CCTCACAATA  CCGCAGAGTC  TAGACTCGTG  GTGGACTTCT
   CTCAATTTTC  TAGGGGGATC  TCCCGTGTGT  CTTGGCCAAA
   ATTCGCAGTC  CCCAACCTCC  AATCACTCAC  CAACCTCCTG
   TCCTCCAATT  TGTCCTGGTT  ATCGCTGGAT  GTGTCTGCGG
   CGTTTTATCA  TATTCCTCTT  CATCCTGCTG  CTATGCCTCA
   TCTTCTTATT  GGTTCTTCTG  GATTATCAAG  GTATGTTGCC
   CGTTTGTCCT  CTAATTCCAG  GATCAACAAC  AACCAGTACG
   GCACCATGCA  AAACCTGCAC  GACTCCTGCT  CAAGGCAACT
   CTATGTTTCC  CTCATGTTGC  TGTACAAAAC  CTACGGATGG
```

```
AAATTGCACC  TGTATTCCCA  TCCCATCGTC  CTGGGCTTTC
GCAAAATACC  TATGGGAGTG  GGCCTCAGTC  CGTTTCTCTT
GGCTCAGTTT  ACTAGTGCCA  TTTGTTCAGT  GGTTCGTAGG
GCTTTCCCCC  ACTGTTTGGC  TTTCAGCTAT  ATGGATGATG
TGGTATTGGG  GGCCAAGTCT  GTACAGCATC  GTGAGTCCCT
TTATACCGCT  GTTACCAATT  TTCTTTTGTC  TCTGGGTATA
CATTTAAGGC  CT-3'
```

11. Procédé selon la revendication 1, caractérisé en ce que ledit plasmide est choisi parmi
pBSAG5 (NRRL B-18028),
pBSAG5I, comme représenté sur la figure 11; et
pBSAGI5I (NRRL B-18021), comme représenté sur la figure 11, dans lequel C sur la figure 11 correspond à un site de restriction *Cla* I et dans lequel pBSAG5 et pBSAG5I ont une orientation différente du fragment *Cla* I.

12. Culture à peu près pure d'une souche du genre *Pichia* transformé à l'aide d'un plasmide choisi parmi pBSAG5 (NRRL B-18028), pBSAG5I, et pBSAGI5I (NRRL B-18021) tels que définis dans la revendication 11.

13. Culture à peu près pure selon la revendication 12, dans laquelle ladite souche est du genre *Pichia pastoris*.

14. Culture à peu près pure d'une souche du genre *Pichia* transformé à l'aide d'un vecteur d'expression comportant le fragment d'ADN tel que défini dans l'une quelconque des revendications 1 à 10.

15. Procédé selon la revendication 1, dans lequel ladite souche de *Pichia transformé* capable de croître sur du méthanol en tant que source de carbone et d'énergie est le *Pichia pastoris* GS 115.

16. Procédé selon la revendication 1, dans lequel ladite souche de *Pichia transformé* capable de croître sur du méthanol en tant que source de carbone et d'énergie est le *Pichia pastoris* GS 190.

17. Procédé selon la revendication 1, dans lequel ladite souche de *Pichia transformé* est du *Pichia pastoris* PPF 1.

18. Procédé selon la revendication 1, caractérisé en ce que :
(a) on cultive une souche de levure transformée à l'aide du plasmide de la revendication 1 dans un milieu nutritif qui est constitué au moins d'une source de carbone réprimant les catabolites et d'une source d'énergie, et
(b) on soumet le produit de l'étape (a) à des conditions de privation de source de carbone.

19. Procédé selon l'une quelconque des revendications 1 à 18 comportant en outre l'isolation et la purification dudit antigène de surface de l'hépatite B.

20. Procédé pour préparer un plasmide utile pour la construction des vecteurs d'expression pour la production de protéines hétérologues sous le contrôle d'une région régulatrice de l'alcool primaire oxydase du *Pichia* (AOX1) pouvant être obtenue à partir du clone pPG4.0 (NRRL B-15868) à partir du site de restriction 5'-*Eco*RI vers le site de restriction 3'-*Sal*I dans lequel le démarrage du plasmide pPG25 contenant ladite région régulatrice est traité comme décrit dans l'exemple II de la description pour obtenir le plasmide pAOP2, comme décrit sur la figure 4.

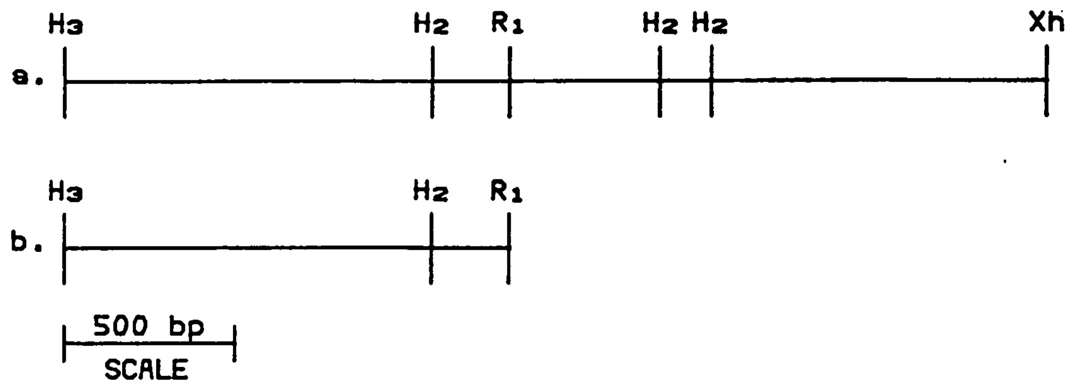


FIG. 1

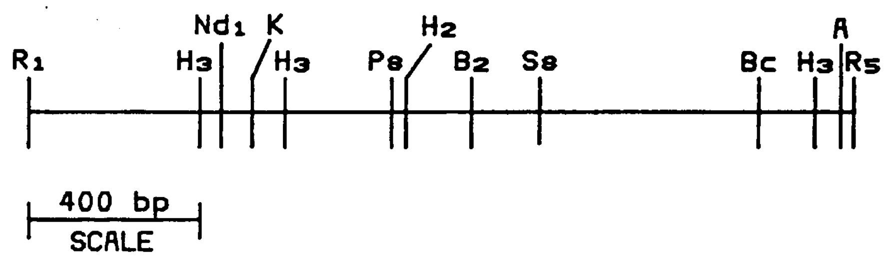


FIG. 2

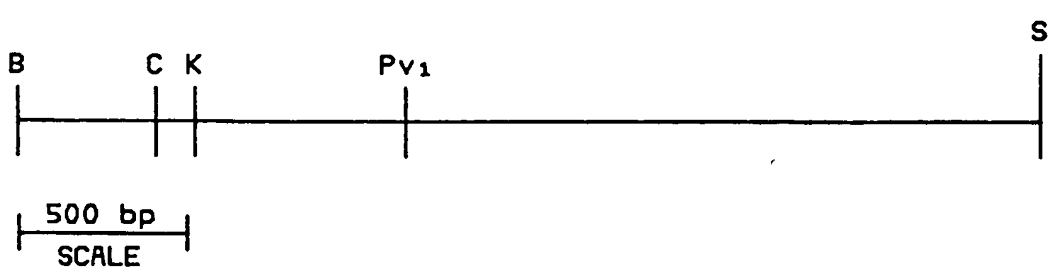


FIG. 3

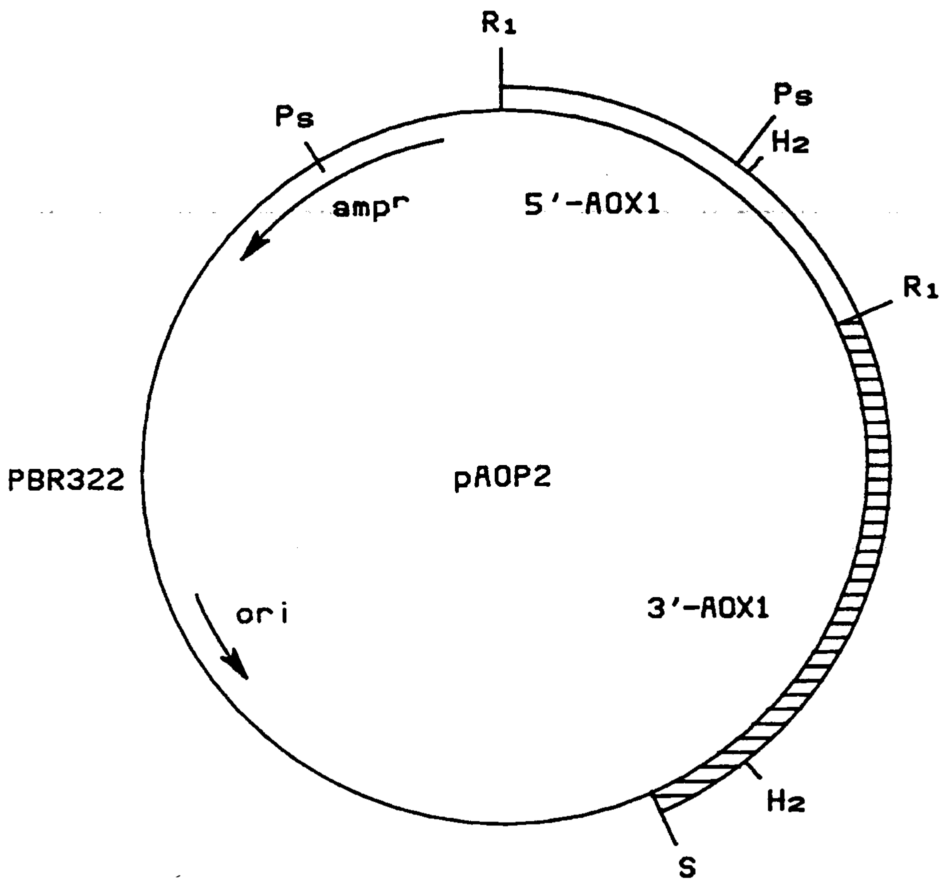


FIG. 4

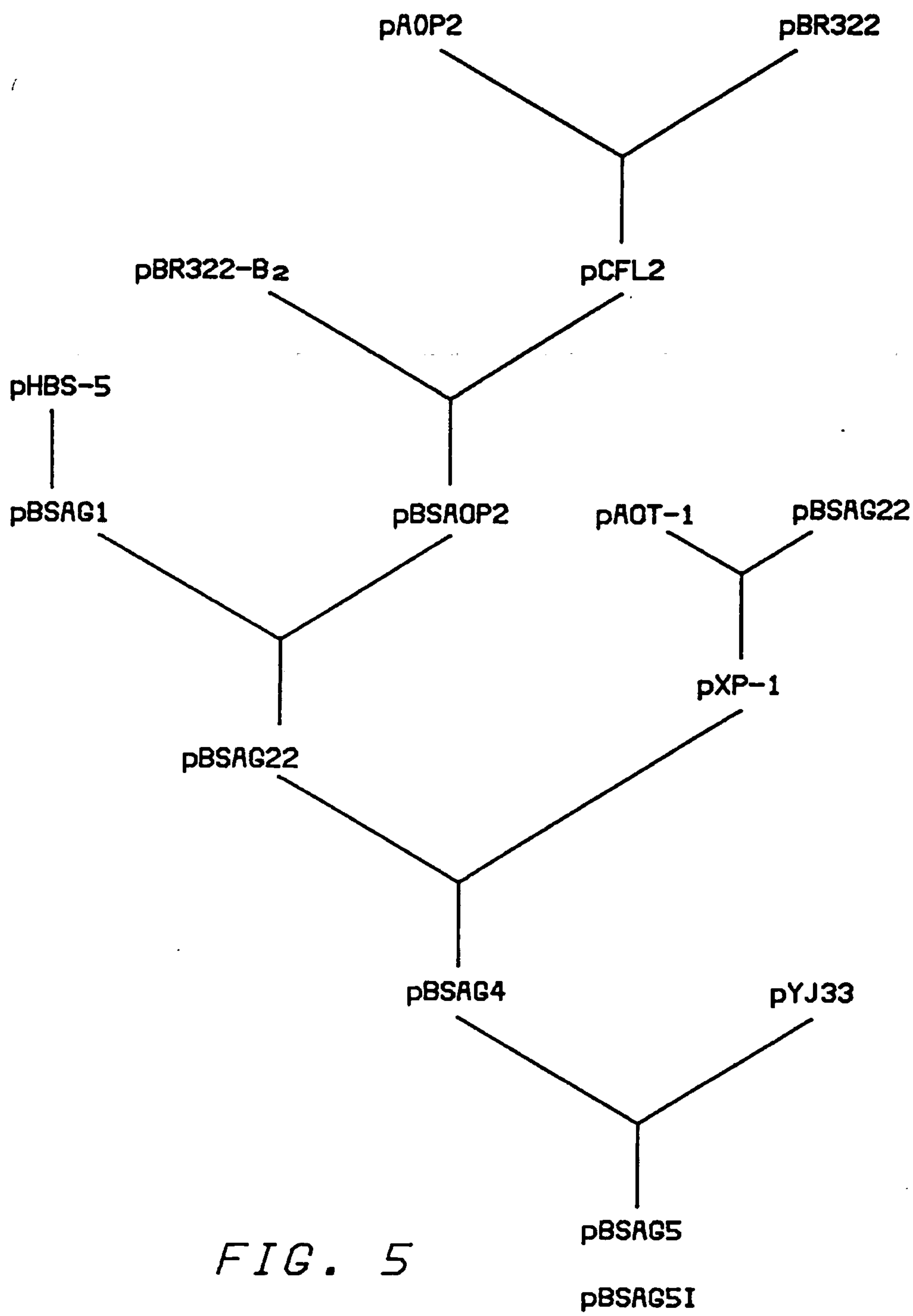

pAOP2
pBR322
pBR322-B2
pCFL2
pHBS-5
pBSAG1
pBSAOP2
pAOT-1
pBSAG22
pXP-1
pBSAG22
pBSAG4
pYJ33
pBSAG5
pBSAG5I

FIG. 5

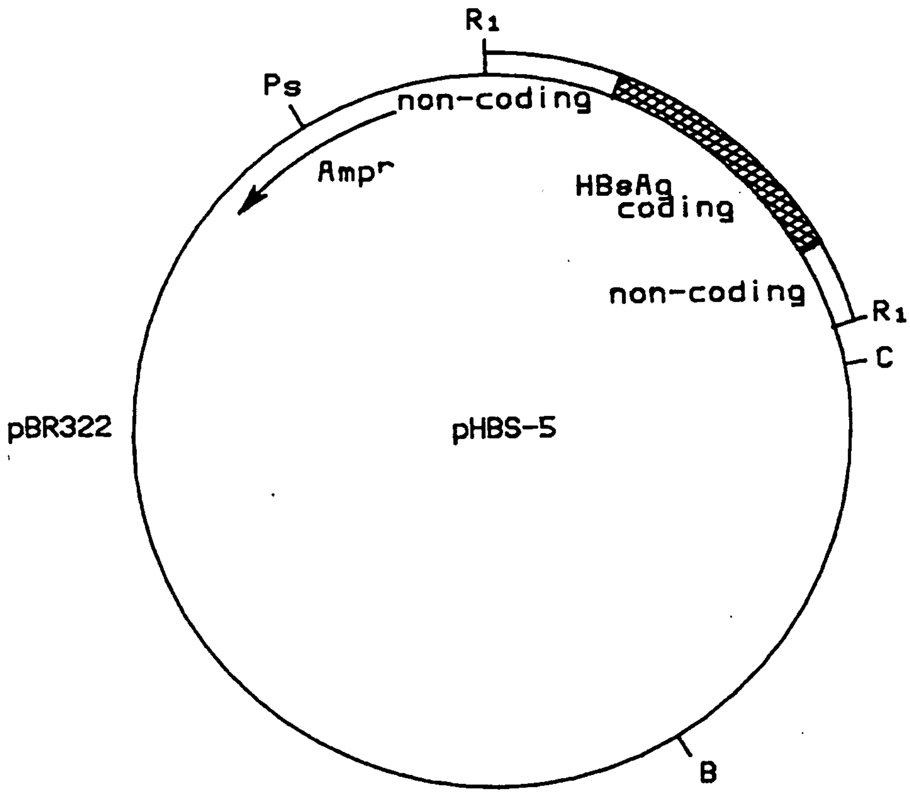

R1
Ps
non-coding
Ampr
HBeAg
coding
non-coding
R1
C
pBR322
pHBS-5
B

FIG. 6

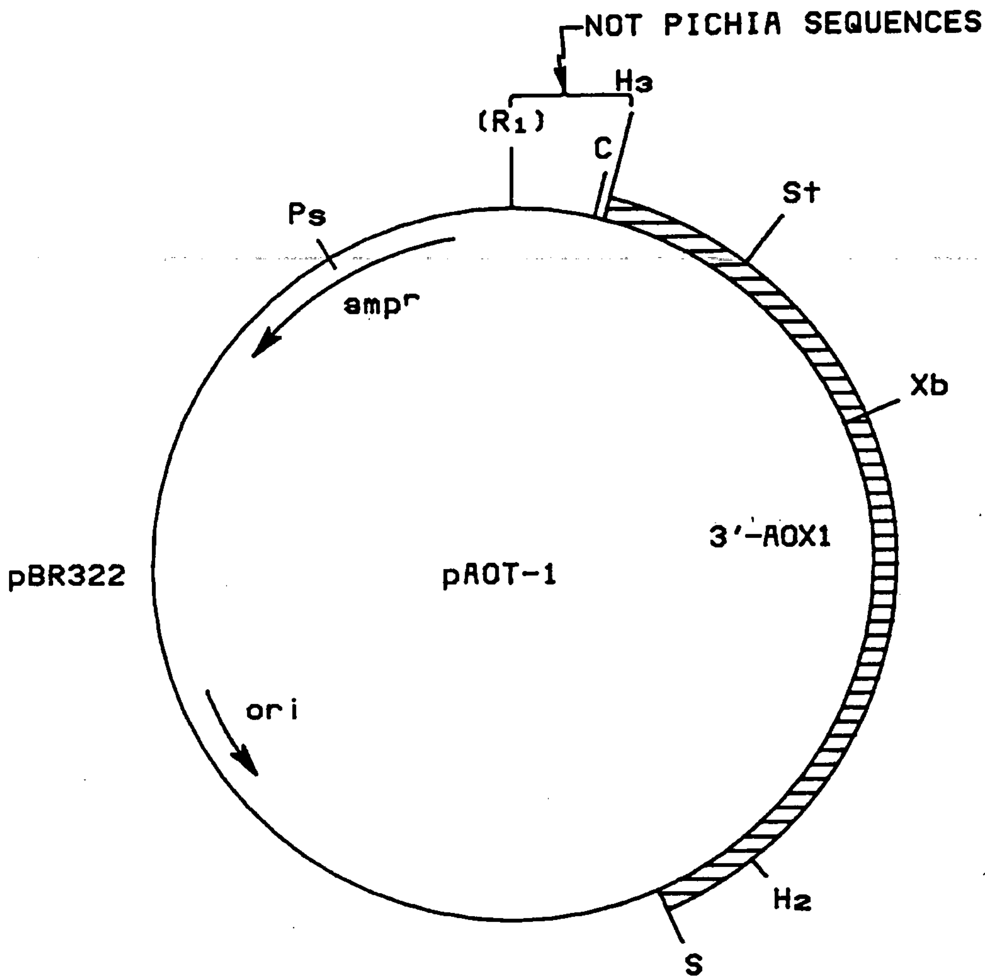

NOT PICHIA SEQUENCES
H3
(R1)
C
St
Ps
ampr
Xb
3'-AOX1
pBR322
pAOT-1
ori
H2
S

FIG. 7

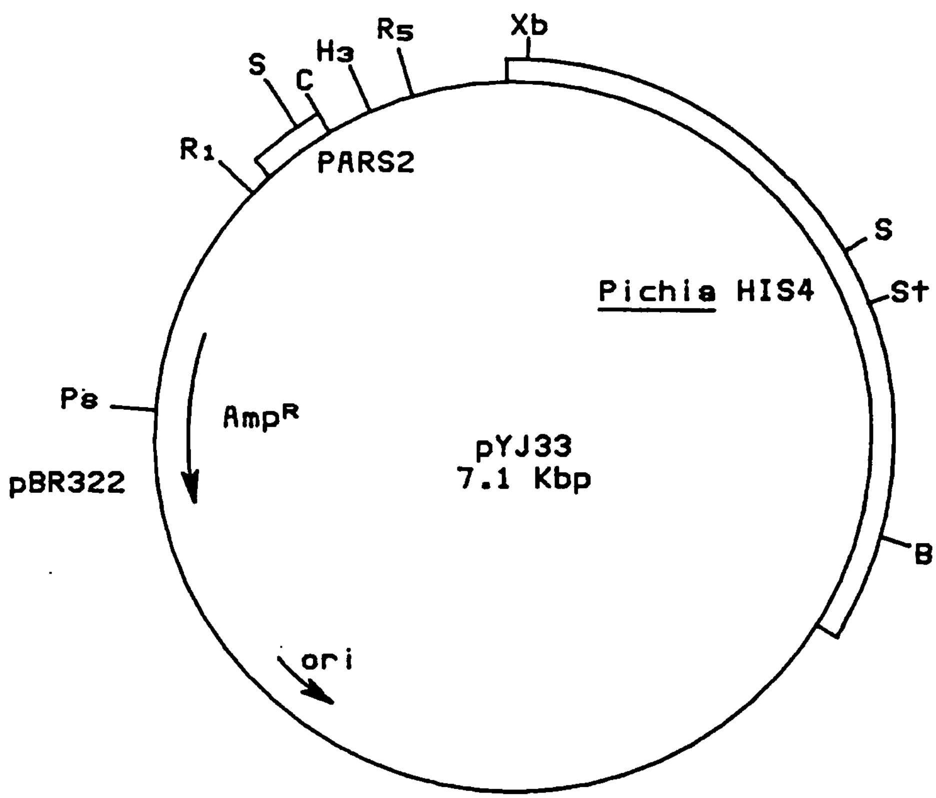
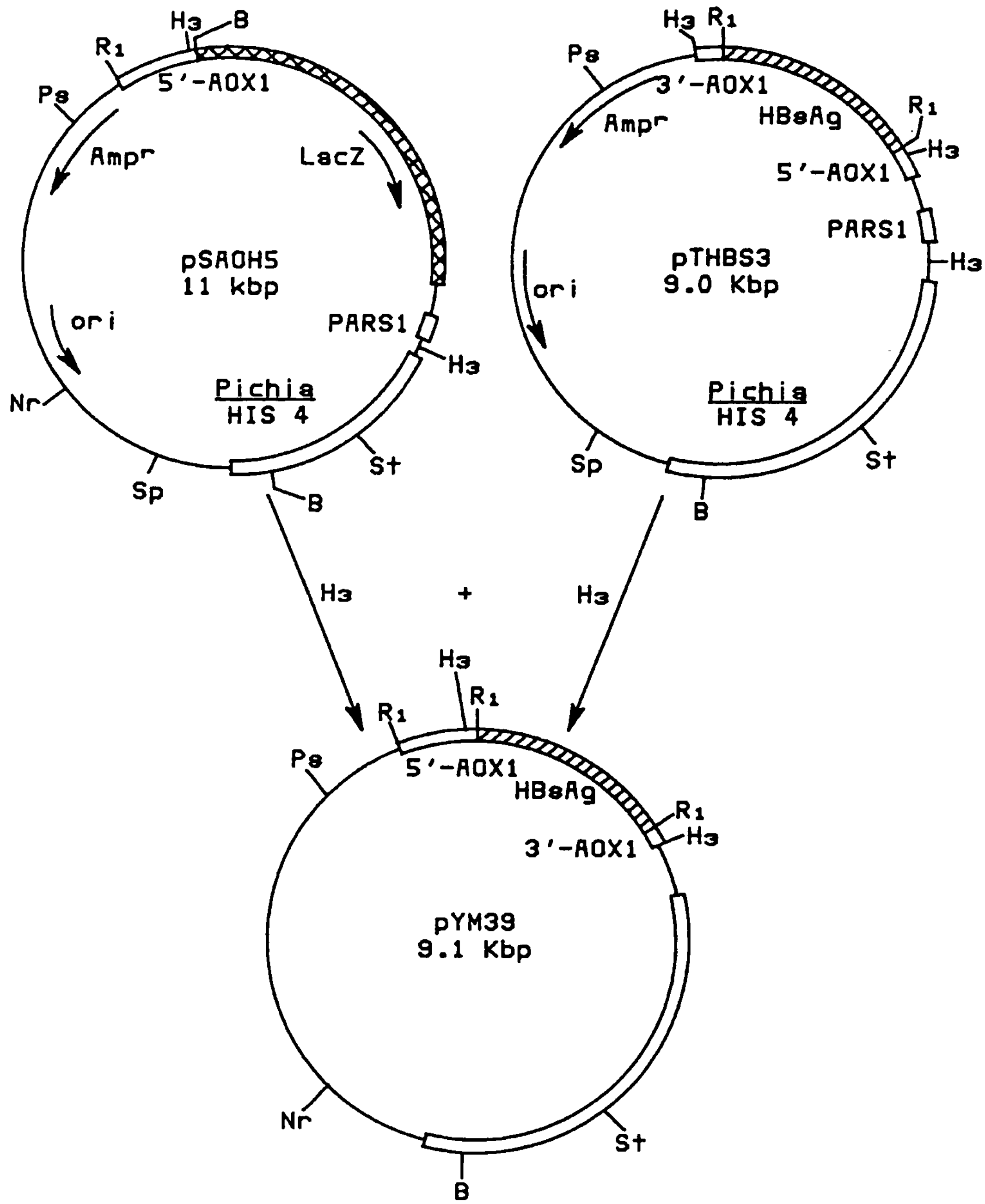

Xb
R5
H3
S
C
R1
PARS2
S
Pichia HIS4
St
Ps
AmpR
pBR322
pYJ33
7.1 Kbp
B
ori

FIG. 8



H3
B
R1
Ps
5'-AOX1
Ampr
LacZ
pSAOH5
11 kbp
ori
PARS1
H3
Nr
Pichia
HIS 4
Sp
St
B
H3
R1
Ps
3'-AOX1
Ampr
HBsAg
R1
H3
5'-AOX1
PARS1
H3
pTHBS3
9.0 Kbp
ori
Pichia
HIS 4
Sp
St
B
H3
+
H3
H3
R1
R1
Ps
5'-AOX1
HBsAg
R1
H3
3'-AOX1
pYM39
9.1 Kbp
Nr
St
B

FIG. 9

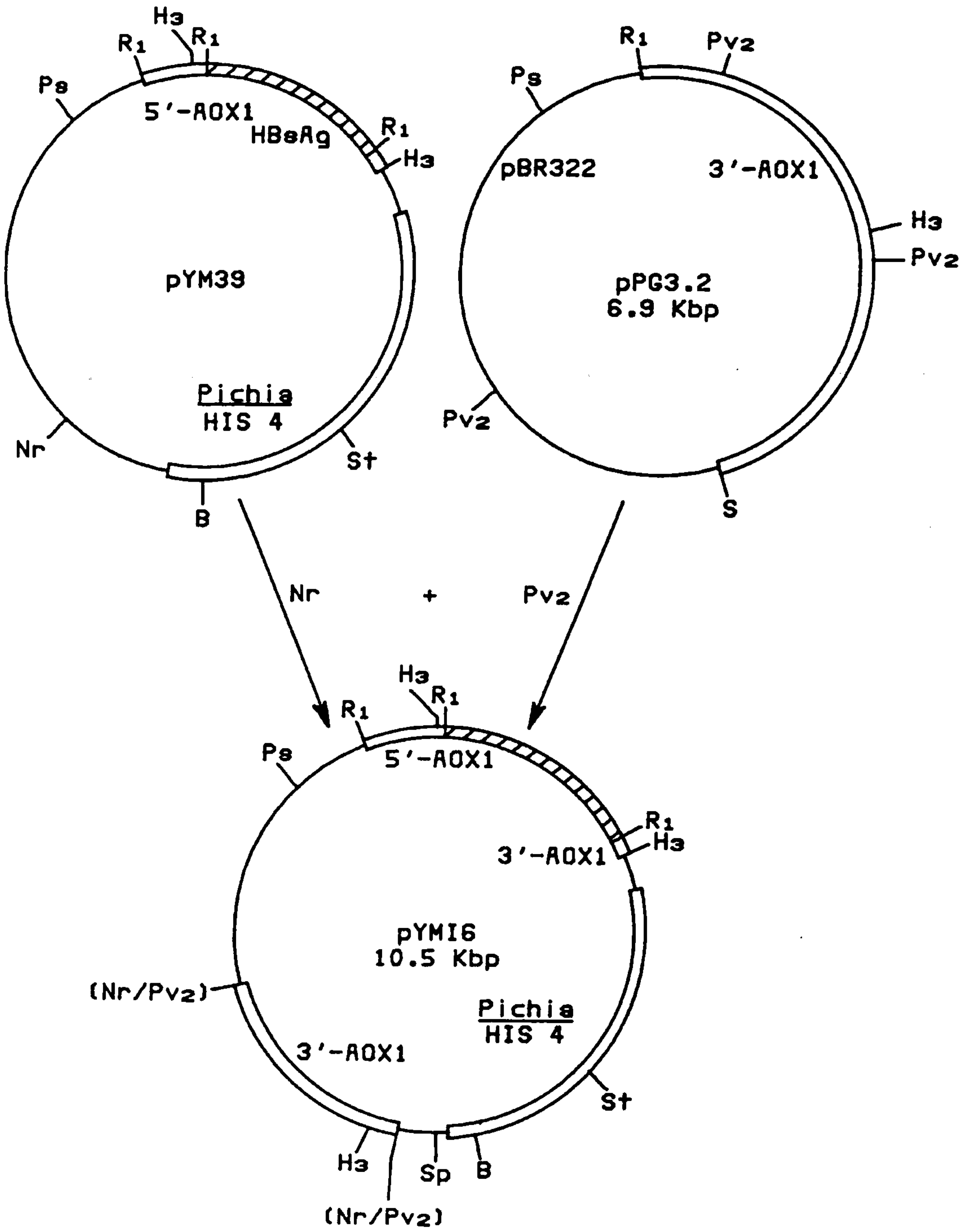

H3
R1
R1
Ps
5'-AOX1
HBsAg
R1
H3
pYM39
Pichia
HIS 4
Nr
St
B
R1
Pv2
Ps
pBR322
3'-AOX1
H3
Pv2
pPG3.2
6.9 Kbp
Pv2
S
Nr
+
Pv2
H3
R1
R1
Ps
5'-AOX1
R1
H3
3'-AOX1
pYMI6
10.5 Kbp
(Nr/Pv2)
Pichia
HIS 4
3'-AOX1
St
H3
Sp
B
(Nr/Pv2)

FIG. 10

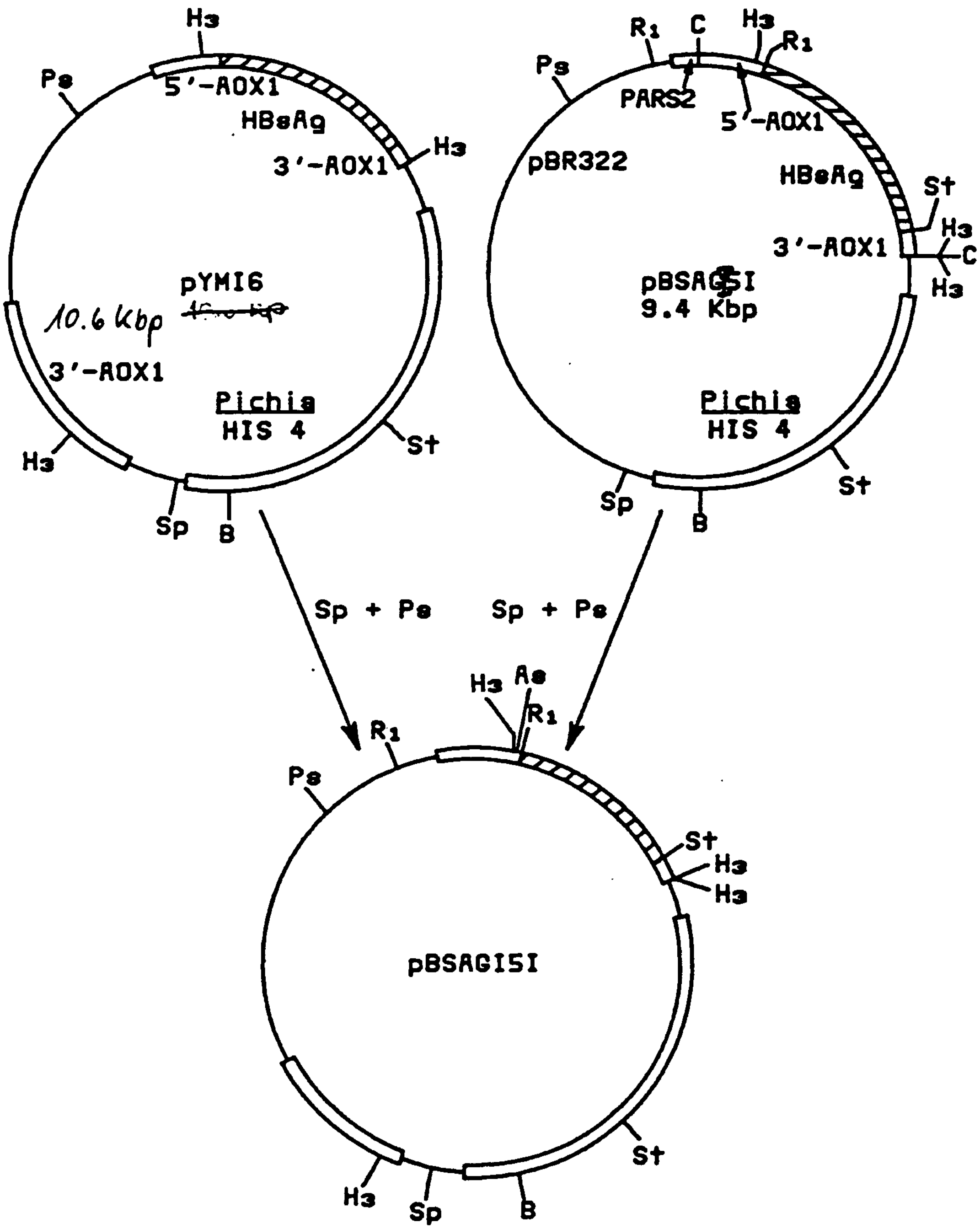

H3
Ps
5'-AOX1
HBsAg
3'-AOX1
H3
pYMI6
10.6 Kbp
3'-AOX1
Pichia
HIS 4
St
H3
Sp
B
R1
C
H3
R1
Ps
PARS2
5'-AOX1
pBR322
HBsAg
St
H3
3'-AOX1
C
H3
pBSAG5I
9.4 Kbp
Pichia
HIS 4
St
Sp
B
Sp + Ps
Sp + Ps
H3
As
R1
R1
Ps
St
H3
H3
pBSAGI5I
St
H3
Sp
B

FIG. 11

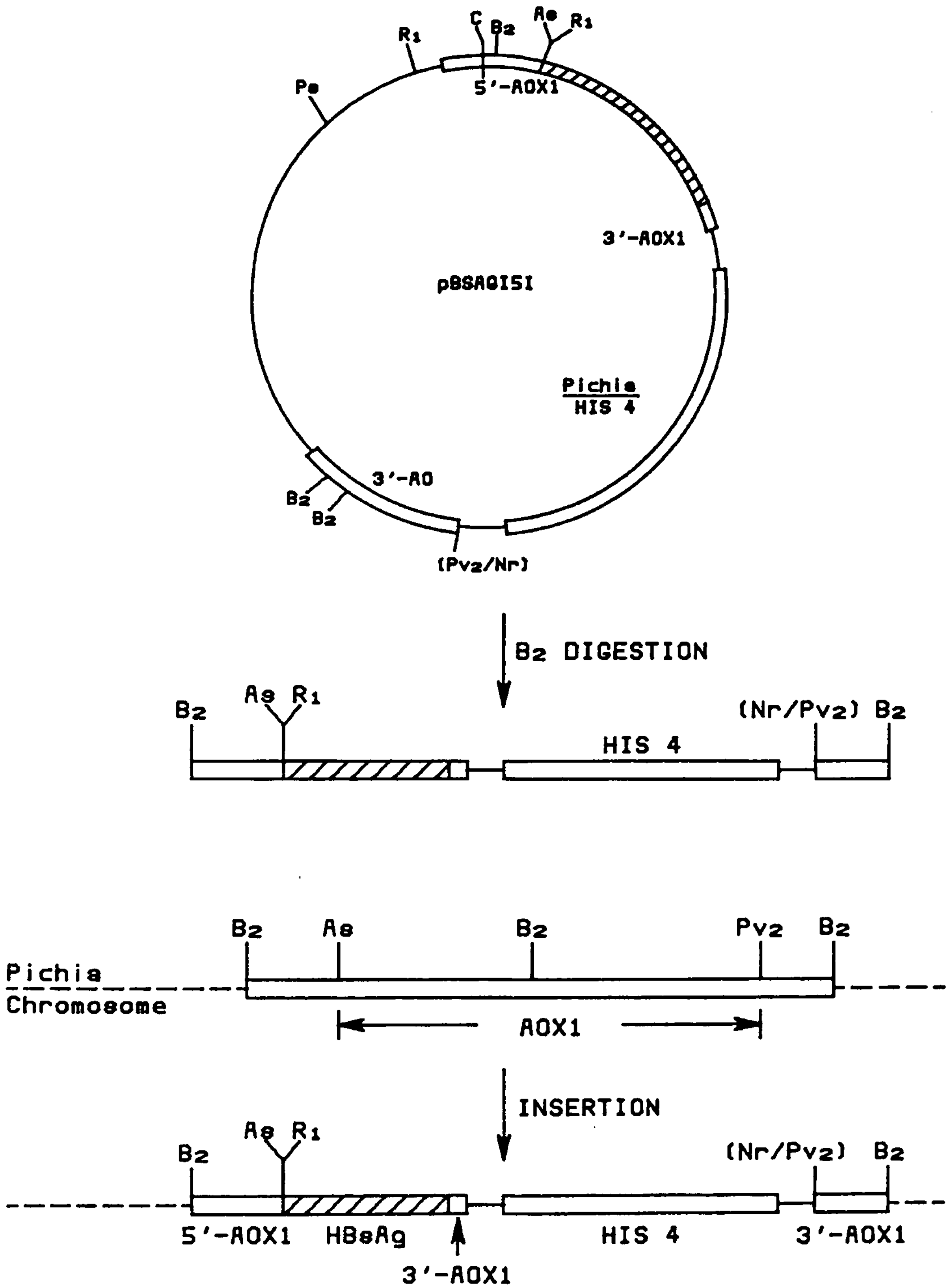

C
B2
As
R1
R1
Ps
5'-AOX1
3'-AOX1
pBSAGI5I
Pichia
HIS 4
3'-AO
B2
B2
(Pv2/Nr)
B2 DIGESTION
B2
As R1
(Nr/Pv2) B2
HIS 4
B2
As
B2
Pv2
B2
Pichia
Chromosome
AOX1
INSERTION
As R1
B2
(Nr/Pv2) B2
5'-AOX1
HBsAg
HIS 4
3'-AOX1
3'-AOX1

FIG. 12

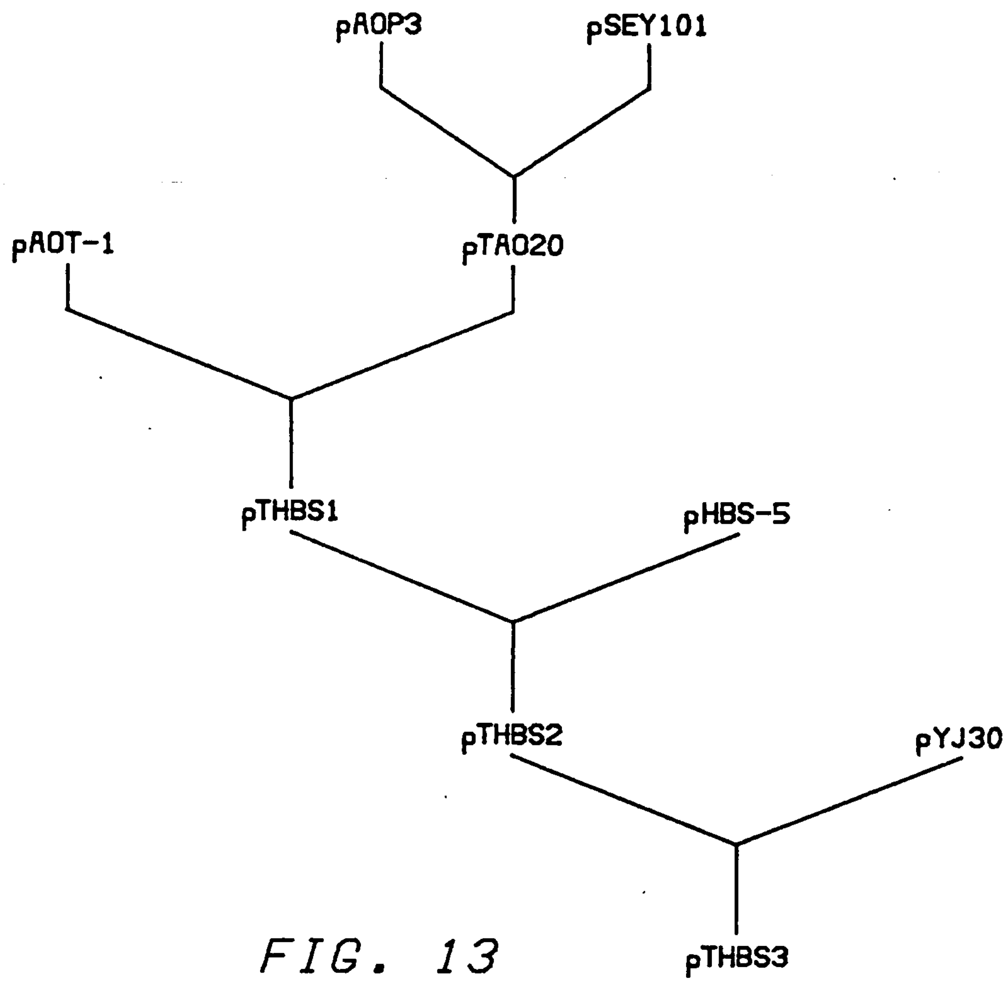

pAOP3
pSEY101
pAOT-1
pTAO20
pTHBS1
pHBS-5
pTHBS2
pYJ30
pTHBS3

FIG. 13